

5-HYDROXYTRYPTAMINE AND NEUROPEPTIDES IN THE RETINA

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Lund 1983

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To my family

This thesis is based on the following papers:

- I. Ehinger, B., Hansson, Ch., and Tornqvist, K.: 5-hydroxytryptamine in the retina of some mammals. *Exp. Eye Res.* 33:663-672, 1981.
- II. Ehinger, B., Florén, I. and Tornqvist, K.: Autoradiography of (³H)-5-hydroxytryptamine uptake in the retina of some mammals. *Graefe's Arch Clin Exp Ophthalmol.* 218:1-8, 1982.
- III. Tornqvist, K., Hansson, Ch., and Ehinger, B.: Immunohistochemical and quantitative analysis of 5-hydroxytryptamine in the retina of some vertebrates. *Neurochem. Int.*, in press, 1983.
- IV. Tornqvist, K., Lorén, I., Håkanson, R. and Sundler, F.: Peptide-containing neurons in the chicken retina. *Exp. Eye Res.* 33:55-64, 1981.
- V. Tornqvist, K., Uddman, R., Sundler, F. and Ehinger, B.: Somatostatin and VIP neurons in the retina of different species. *Histochemistry*, 76:137-152, 1982.
- VI. Tornqvist, K. and Ehinger, B.: Glucagon immunoreactive neurons in the retina of different species. *Graefe's Arch Clin Exp Ophthalmol.* in press, 1983.
- VII. Tornqvist, K. and Ehinger, B.: Simultaneous immunohistochemistry and autoradiography of peptides and 5-hydroxytryptamine in bird retina. *Neurochem. Int.*, in press, 1983.
- VIII. Ekman, R. and Tornqvist, K.: Glucagon and VIP in the retina. Manuscript 1983.

The papers will be referred to in the text by their Roman numerals.



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1. INTRODUCTION

In the retina there are different types of neuronal systems, photoreceptors, bipolar cells, ganglion cells, horizontal cells, amacrine cells and interplexiform cells. The nerve impulse is generated in the photoreceptors and transferred via bipolar cells to ganglion cells which by their long axons bring the impulse to the central nervous system. The signal is modulated in the outer plexiform layer by horizontal cells and in the inner plexiform layer by amacrine cells. The interplexiform cells are thought to bring information centrifugally from the inner to the outer plexiform layer. All these neurons use several different substances as their transmitter and there are also considerable differences between different animal species (Ehinger, 1982). At least some of the photoreceptors are thought to use aspartate or glutamate as their transmitter. The transmitters of bipolar cells and mammalian horizontal cells remain unclear, whereas GABA is a likely transmitter in a population of horizontal cells in goldfish, frog, chicken, and pigeon. Some interplexiform cells most certainly use dopamine as their transmitter, whereas GABA and glycine are possible transmitter candidates in other types of such cells. In the ganglion cells again, the transmitters are less well established though aspartate, glutamate or acetylcholine are possible candidates in a fraction of ganglion cells. In the sixth cell type, the amacrine cells, a large number of possible transmitter candidates such as acetylcholine, GABA, glycine, dopamine, a still unknown indoleamine and several peptides have been identified (Ehinger, 1982). The GABA and glycine neurons are of the diffuse type, whereas all

the others seem to constitute groups of stratified amacrine cells (Ehinger, 1982).

The indoleamine accumulating amacrine cells have been described in the retina of rabbit, cat, goldfish, river lamprey, chicken, pigeon, and Cebus monkey (Ehinger and Florén, 1976; Ehinger et al., 1977; Florén, 1979a; Dowling et al., 1980). They have also been noted in the frog retina (Ehinger, unpublished observations). Their transmitter has been debated. 5-hydroxytryptamine is an indoleamine and also a transmitter in the central nervous system, and has consequently been suggested to have a transmitter function also in the retina. However, this has for several reasons been regarded as less probable in mammals. No endogenous 5-hydroxytryptamine has been found with formaldehyde fluorescence (Florén, 1979b); the 5-hydroxytryptamine content in at least the mammalian retina is very low and cannot be increased with pharmacological treatment (Florén and Hansson, 1980); there is in the rabbit retina no detectable amounts of tryptophan hydroxylase, the rate limiting enzyme in the synthesis of 5-hydroxytryptamine, (Florén and Hansson, 1980); the indoleamine accumulating neurons in rabbit retina are not destroyed by p-chloroamphetamin as is the case with 5-hydroxytryptamine containing neurons in the brain (Florén, 1979b) and, finally, the uptake system for indoleamines in the rabbit retina is different from that in the hypothalamus (Florén, 1979b).

Recently, a number of peptides have emerged as putative neurotransmitters in the central and peripheral nervous systems (Hökfelt et al., 1980; Håkanson et al., 1981) and also in the retina (review: Brecha and Karten, 1983). The immunoreactive peptides to date found or suggested to be present in the

retina are listed in Table 1. They are with few exceptions localized among the amacrine cells, which means that more than a dozen putative neurotransmitters have been found in such cells. The rich variety of transmitter substances within this cell type has lead to the question whether more than one transmitter substance occur within the same neuron as has been found in the central nervous system (Hökfelt et al., 1978; Johansson et al., 1981). However, no such co-occurrence has been found in the retina so far.

TABLE 1

Immunoreactive neuropeptides in the retina

SOMATOSTATIN	CHOLECYSTOKININ
VIP (vasoactive intestinal polypeptide)	LH-RH
GLUCAGON	TRH?
ENKEPHALIN	APP?
NEUROTENSIN	ACTH???
SUBSTANCE P	Beta-endorphin??

The aim of this study has been to extend previous studies on the indoleamine accumulating neurons in the retina and their transmitter as well as to investigate peptide immunoreactive retinal neurons. Possible co-occurrence of 5-hydroxytryptamine and the peptides somatostatin, glucagon, substance P, neurotensin and enkephalin has been investigated by means of simultaneous autoradiography and immunohistochemistry. To get further information on immunoreactive VIP and glucagon, the morphological findings have been supplemented by chemical studies.

2. MATERIALS AND METHODS

2.1 ANIMALS USED

Eyes from the following animals were used: baboon (Papio papio), cow, pig, cat, rabbit, guinea-pig, rat, mouse, pigeon (Columba livia), chicken, frog (Rana temporaria), and goldfish (Carassius auratus) of 10-15 cm length. In addition, specimens from one Cynomolgus monkey (Macaca fascicularis) and one squirrel monkey (Saguineus oedipus) were used. All animals were light adapted and exposed to ordinary laboratory illumination (about 200 lux). Retinae from cow and pig were obtained in a nearby slaughter-house within two or three minutes of the slaughter whereas the other animals were killed in the laboratory. Human retinae were obtained from eight patients, age 48-80 years, all enucleated because of malignant melanoma. Pieces of retina were taken well away from the tumour.

2.2 SUMMARY OF HISTOCHEMICAL METHODS

2.2.1 Formaldehyde fluorescence histochemistry

Pieces of normal retina or pieces that had previously been incubated in 5,6-dihydroxytryptamine (1, 5, 10, 25 or 50 µg/ml) or alpha-methylnoradrenalin (1, 5 or 10 µg/ml) were used. Incubation was performed either at room temperature for 30-45 minutes or at +37°C for 15 minutes. Both methods were used for both substances and all concentrations. The specimens were then rinsed for 2 x 5 minutes in ice-cold balanced salt solution (Ames et al., 1980) and subsequently processed according to the standard Falck and Hillarp technique (see Falck and Owman, 1965, or Björklund et al., 1972). The same technique was also applied to pigs which had received intraocular injections of 20 or 50 µg of 5,6-dihydroxytryptamine 4 hours before processing the retina. The tissue samples were freeze-dried, exposed to formaldehyde for 1 hour at +80°C (whereby

the fluorophores were formed), embedded in vacuo in paraffin wax and sectioned. The sections were examined in a fluorescence microscope with conventional filter settings whereby dopamine show greenish and indoleamines yellowish fluorescence.

2.2.2 Autoradiography

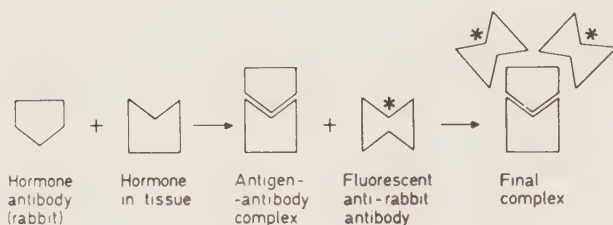
Retinae from cow and pig were incubated in varying concentrations (1.2 or 2.6 $\mu\text{Ci/ml}$) of (^3H)-5-hydroxytryptamine either at room temperature or at $+37^\circ\text{C}$ according to the procedures described above, and subsequently freeze-dried. The specimens were then fixed in gaseous formaldehyde for 1 hour at $+80^\circ\text{C}$, embedded in plastic (Durcopan^(R), Fluka) in vacuo and sectioned. The sections were then covered with stripping film (Kodak AR 10) floated on distilled water and exposed for 4-12 weeks. The emulsion was developed in a standard commercial developer (Kodak D 19) and fixed in Kodafix solution.

2.2.3 Immunohistochemistry

The specimens were fixed by immersion in 0.1 M phosphate buffer containing 4 % paraformaldehyde (pH 7.2) for 24 hours and rinsed for 24-48 hours in 0.1 M phosphate buffer containing 5 or 30% sucrose. The specimens were then frozen on dry ice and sectioned (15 μm) in a cryostat at -20° . The sections were processed for the immunohistochemical demonstration of peptides or 5-hydroxytryptamine using the indirect immunofluorescence method according to Coons et al. (1955) or the peroxidase-antiperoxidase (PAP) technique according to Sternberger (1979).

In the indirect immunofluorescence technique (Fig. 1), the sections were incubated with the peptide or 5-hydroxytryptamine antiserum for 3 hours at room temperature in a moist chamber. After thorough rinsing, the site of the antigen-antibody reaction was revealed with fluorescein isothiocyanate labelled anti-rabbit Ig G, applied for 30 min at room temperature. The sections were examined in a fluorescence microscope with special filters selected to give peak excitation at 450-490 nm and barrier filters with a cut-off edge at 515 nm (Leitz I2 filter system).

IMMUNOFLUORESCENCE TECHNIQUE



IMMUNOPEROXIDASE (PAP) TECHNIQUE

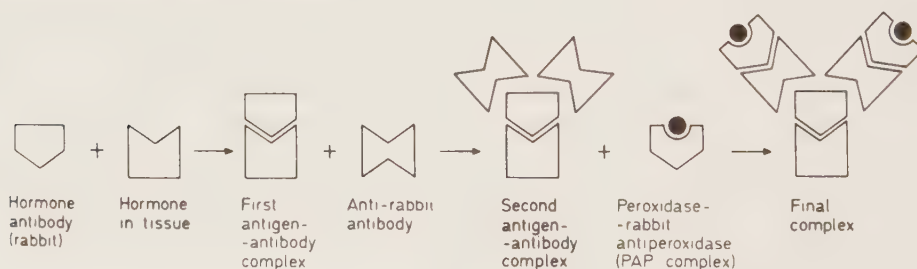


Fig. 1. Schematic drawing illustrating the indirect immunofluorescence method (above) and the peroxidase-anti-peroxidase procedure (below).

In the peroxidase-antiperoxidase procedure (Fig. 1), the sections were incubated with the peptide antiserum overnight at +4°C. After rinsing, the sections were incubated with unlabelled sheep anti-rabbit Ig G for 30 min at room temperature followed by incubation for 30 min with PAP complex, i. e. horseradish peroxidase bound to a rabbit antibody raised against peroxidase. The enzyme was developed with diaminobenzidine hydrochloride and hydrogen peroxide. For immunohistochemistry combined with autoradiography the sections were left unmounted, whereas for other purposes they were mounted in Permount^(R).

In one study (paper V), whole mount preparations were used. Pieces of retina were stretched on microscope slides, fixed in a solution of formaldehyde and picric acid, dehydrated, cleared in xylene and hydrated in a series of ethanol solutions (Costa et al., 1980). The whole mounts were then processed for the immunohistochemical demonstration of somatostatin or VIP using the indirect immunofluorescence method (Coons et al., 1955). Some of the whole mounts were subsequently washed, incubated with PAP complex and stained for peroxidase (Sternberger, 1979).

2.2.4 Simultaneous autoradiography and immunohistochemistry

Chicken and pigeon received intraocular injections of (³H)-5-hydroxytryptamine (35 or 40 µCi). Four hours later the retinæ were dissected out, fixed, rinsed, and sectioned as for immunohistochemistry. The PAP technique was then applied to the sections and those with good PAP staining were further processed for autoradiography, i.e. covered with stripping film (Kodak AR 10) floated on distilled water and exposed for

TABLE 2

Antigen	Code	Raised against	Directed against	Working Immuno-fluoresc.	dilution PAP-staining	Source	References
1. Glucagon	7811	unconj. porcine glucagon	midportion	1:160	1:640	Milab, Malmö, Sweden	Lorén et al, 1979
	4304	protein-conj. porcine glucagon	midportion	1:80		J Holst, Copenhagen Denmark	Holst and Aasted, 1974
2. Leu-enkephalin	"leu-enk"	protein-conj. leu-enkephalin		1:60		RJ Miller and KJ Chang Burroughs Wellcome Res. Lab., USA	Alumets et al, 1978
	47169	protein-conj. leu-enkephalin		1:100	1:1000	Immuno Nuclear Corp. Stillwater, USA	
3. Neurotensin	HC-8	protein-conj. synth. bovine neurotensin	C-terminal hexapeptide	1:40		R Carraway, Boston Mass., USA	Carraway and Leeman, 1976
	47189	protein-conj. neurotensin		1:100	1:500	Immuno Nuclear Corp. Stillwater, USA	
4. Somatostatin	19578	protein-conj. synth. somatostatin		1:160		MP Dubois, Nouzilly France	Dubois, 1975
	29189	protein-conj. synth. somatostatin		1:80		Immuno Nuclear Corp. Stillwater, USA	
	31100	protein-conj. synth. somatostatin		1:80	1:500	" "	
5. Substance P	14411	protein-conj. substance P		1:80	1:500	" "	
6. VIP	7852	unconj. VIP	VIP 1-15	1:160		Milab, Malmö, Sweden	Alm et al, 1980
7. 5-hydroxy-tryptamine	18240	protein-conj. 5-hydroxy-tryptamine		1:200 1:500		Immuno Nuclear Corp. Stillwater, USA	Steinbusch et al, 1978

4, 6, 8 or 12 weeks. The emulsion was developed and fixed as in standard autoradiography.

2.2.5 Antisera

Details on the antisera are given in Table 2.

2.2.6 Absorption controls

5-hydroxytryptamine antiserum diluted 1:500 was preincubated for 36 hours at +4°C in 30 mM solutions of each of the following substances: 5-hydroxytryptamine, 5,7-dihydroxytryptamine, 5,6-dihydroxytryptamine, 6-hydroxytryptamine, bufotenine, 5-methoxytryptamine, 5-methoxytryptophan, 5-hydroxytryptophan, melatonin, L-tryptophan, and norharmane.

Peptide antisera in the appropriate dilutions were preincubated for 24 hours with the respective antigen in excess (10-100 µg/ml diluted antiserum). Only staining abolished by this treatment was regarded as specific.

2.2.7 Cross reactivity

Each antibody recognizes a short sequence of amino acids on its antigen. It cannot be excluded that the antiserum also demonstrates other peptides containing the immunoreactive sequence of amino acid residues. To test their specificity, antisera were therefore exposed to a number of structurally related and unrelated peptides, usually 10-100 µg/ml diluted antiserum. The glucagon antisera used (codes no. 7811 and 4304) are known to cross-react with gut-type glucagon (glicentin) but not with other related peptides (Lorén et al., 1979). The antisera against leu-enkephalin (codes "leu-enk" and 47169) are known to cross-react with met-enkephalin (Alumets et al., 1978). The neurotensin and somatostatin antisera are

not known to cross-react with other peptides (Dubois, 1975; Carraway and Leeman, 1976). The substance P antiserum cross-reacts with physalaemin and eledoisin (Ehinger et al., 1983). The VIP antiserum (code no. 7852) cross reacts only insignificantly (0.1% or less) with CCK, GIP, secretin and glucagon. The 5-hydroxytryptamine antiserum was found to cross-react with 5,7-dihydroxytryptamine, 5,6-dihydroxytryptamine, bufotenine and 6-hydroxytryptamine.

2.3 CHEMICAL METHODS

2.3.1 High performance liquid chromatography of 5-hydroxytryptamine

Quantitative studies on 5-hydroxytryptamine in the retina were performed according to the method described by Hansson and Rosengren (1978). Tissue samples were homogenized in 2 ml 0.4 M HClO_4 together with 100 ng of alpha-methyl-5-hydroxytryptamine which served as an internal standard. After centrifugation and adjustment of pH to 6.0, the samples were purified and concentrated by passing them through a weakly acidic cation exchange resin (Amberlite IRP-64). 5-hydroxytryptamine as well as other amines and the internal standard were then eluted with 2 ml 1.2 M HCl. 100 μl of the eluted volume was chromatographed on microparticulate reversed phase packing material with chemically bonded octadecyl groups on 5 μm silica using a high pressure pump and an electrochemical detector. The limit of detection for 5-hydroxytryptamine in this system is about 25 pg.

2.3.2 Extraction of VIP

Retinae were rapidly dissected out, weighed and frozen in liquid nitrogen. VIP was extracted according to Emson et al. (1979), i. e. retinae were placed in boiling 1 M acetic acid

(1 ml/100 mg retina or at least 2 ml) for 10 min, then gently homogenized and centrifuged at 3000g for 10 minutes. The supernatant was collected, freeze-dried and stored at -20°C until radioimmunoassay or gel chromatography.

2.3.3 Radioimmunoassay of VIP

Radioimmunoassay was performed with a VIP antiserum (code no. 7852) raised in rabbits. Antiserum was incubated for three days at +4°C with standard, tissue extracts or column eluates and then with (^{125}I)-VIP for another two days at +4°C. Bound and free VIP were separated by using a second antibody. Each tissue extract was tested in serial dilutions to check that it diluted in parallel with the standard curve.

2.3.4 Gel chromatography of VIP

The freeze-dried retinal extracts were reconstituted in 0.25 M ammonium acetate buffer, pH 6.5, containing 72.5 μmol bovine serum albumin/l. Chromatography was performed on a Sephadex^(R) G-50 superfine column (1,6x100 cm) which was calibrated with Blue Dextran and (^3H)-glycine. The same buffer as used for reconstitution of the sample was used as eluant. The fractions obtained were analyzed for VIP by radioimmunoassay. Samples containing trace amounts of (^{125}I)-labelled VIP were also chromatographed and the elution patterns were compared.

2.3.5 Extraction of glucagon

Glucagon was extracted according to the method described by Larsson et al. (1975). In short, retinae were rapidly dissected out, weighed, frozen in liquid nitrogen and homogenized in 65% acidified ethanol (1 ml/100 mg retina or at least 2 ml). The homogenate was left at +4°C for 4-12 hours. After

centrifugation (3000g, 10 min) the pH of the supernatant was adjusted to 8.0, the resulting precipitate discarded, and ethanol and ether were added to the supernatant. After 12 hours at +4°C, the precipitate was collected, dried and stored at -20°C until radioimmunoassay or gel chromatography.

2.3.6 Radioimmunoassay of glucagon

Glucagon immunoreactivity in tissue extracts and chromatographic fractions was determined as described earlier (Faloona et al., 1974; von Schenk 1977; von Schenk, 1978). 500 µl of antiserum diluted 1:60 000 was incubated first with 200 µl competitor (standard, tissue extracts or column eluates) for 24 hours at +4°C and then for another 72 hours with 500 µl of (¹²⁵I)-glucagon at +4°C. Bound and free (¹²⁵I)-glucagon were separated by adsorption of free (¹²⁵I)-glucagon to dextran-coated charcoal. Each tissue extract was tested at serial dilution to see that it diluted in parallel with the standard curve.

2.3.7 Gel chromatography of glucagon

The dried samples were reconstituted in 0.5 M acetic acid. Gel chromatography was performed on Sephadex^(R) G-50 superfine columns (1.6x35 cm and 1.6x100 cm respectively). The columns were calibrated with Blue Dextran, (²²Na) and (¹²⁵I). 0.5 M acetic acid was used as eluant. The fractions obtained were analyzed for glucagon by radioimmunoassay. Samples containing trace amounts of (¹²⁵I)-glucagon and (¹²⁵I)-glicentin were then chromatographed and the elution patterns were compared.

2.3.8 High performance liquid chromatography of glucagon

For HPLC an instrument from Waters, Ass. (Milford, Mass., USA) with a U6 K injector, two M 6000 A pumps, one 660 solvent programmer, a 440 variable UV-detector and 3.9x300 mm μ -Bondapak C₁₈ column was used. The solvent was composed of acetonitrile/0.1 M phosphate buffer, pH 3.5. A 32-50% linear gradient of CH₃CN was applied for 20 min with a flow rate of 1 ml/min and 1 ml fractions were collected. The concentration of glucagon immunoreactive material in the fractions obtained was then determined with radioimmunoassay.

3. RESULTS AND DISCUSSION

3.1 INDOLEAMINE ACCUMULATING NEURONS

3.1.1 Indoleamine accumulating neurons in goldfish, frog, chicken and pigeon. (III)

3.1.1.1 Goldfish: In the goldfish retina, 5-hydroxytryptamine immunoreactive cell bodies were seen in the innermost two cell rows of the inner nuclear layer, constituting at most a few per cent of the cells in their cell rows. They were rounded when situated in the innermost cell row and more pear-shaped when situated further outwards. They sent processes to two sublayers of nerve fibres in sublaminae 1 and 5 in the inner plexiform layer. The outermost was somewhat more prominent than the innermost and the two sublayers were connected by radially running nerve fibres. At times a third, incomplete sublayer was seen in sublamina 3. These results obtained with immunohistochemistry agree very well with those obtained with formaldehyde fluorescence (Ehinger and Florén, 1976) and autoradiography (Tornqvist, unpublished observations). Furthermore, ultrastructural studies have demonstrated indoleamine accumulating cell bodies in the innermost part of the inner nuclear layer with their terminals confined to two or three

sublayers in the inner plexiform layer (Holmgren-Taylor, 1983). Osborne et al. (1982a) have also very recently obtained similar results with a monoclonal antibody directed against serotonin.

3.1.1.2 Frog: In the frog retina, 5-hydroxytryptamine immunoreactivity was found in two different types of cell bodies in the innermost cell rows of the inner nuclear layer. One type was large and slightly polyhedral and situated in the innermost cell row exclusively, whereas the other type was more rounded, smaller and found in the innermost few cell rows. Both cell types sent processes to a dense plexus of nerve fibres evenly distributed throughout the inner plexiform layer. Short immunoreactive fibres were also seen encircling the ganglion cells, and in the nerve fibre layer. This morphological distribution of cell bodies and nerve fibres agrees very well with the results obtained with formaldehyde histofluorescence studies of 5-hydroxytryptamine accumulation (Ehinger, unpublished observations). The results also agree with those of Osborne et al. (1981a; 1982a) who investigated 5-hydroxytryptamine containing neurons in the frog retina with autoradiography as well as with immunohistochemistry, using a monoclonal serotonin antibody. The number of cell bodies found with autoradiography was higher than with immunohistochemistry. This can perhaps be explained by different concentrations of amines in different cell bodies so that some may escape detection with immunohistochemistry, whereas all neurons possess the ability to accumulate (^3H)-5-hydroxytryptamine.

3.1.1.3 Chicken and pigeon: The distribution of 5-hydroxytryptamine immunoreactive neurons was very similar in chicken and pigeon retina. Ovoid cell bodies were found in the inner-

most cell rows of the inner nuclear layer, constituting approximately 5-10% of the cells in their row. Their processes formed two sublayers in sublaminae 1 and 4 of the inner plexiform layer. The innermost was the most prominent and the two sublayers were connected by radially running nerve fibres.

Ovoid cell bodies with weaker fluorescence than the ones in the innermost cell rows were discerned in the middle of the inner nuclear layer and weakly fluorescent nerve fibres projected inwards and outwards from them. The fibres projecting inwards were hard to observe because of their very weak fluorescence. The fibres projecting outwards reached the outer plexiform layer. In the pigeon retina the fluorescence was more intense than in the chicken and in this case the fibres were seen to form a layer of weakly fluorescent terminals in the outer plexiform layer. The number and distribution of neurons was identical with that of the indoleamine accumulating neurons found with formaldehyde fluorescence (Florén, 1979a), and with the neurons able to take up (^3H)-5-hydroxytryptamine demonstrated by Osborne (1982a) and ourselves (paper VII). This means that in the bird retina not only amacrine cells but also the so called bipolar-like cells seem to contain 5-hydroxytryptamine. As they display a weaker fluorescence than the amacrine cells, their 5-hydroxytryptamine content is probably lower. This hypothesis is also favoured by the fact that Osborne (1982a), using a monoclonal antibody with a different sensitivity, was not able to visualize them. They also accumulate exogenously applied 5-hydroxytryptamine less readily than amacrine cells, as higher concentrations of indoleamines were needed to reveal them in formaldehyde fluorescence studies (Florén, 1979a). Further, in autoradiograms, the bipolar-like cells were less heavily labelled than the

amacrine cells (paper VII). They have their cell bodies in the position of bipolar cells but their signalling direction is doubtful. The fibres projecting inwards were only weakly fluorescent, indicating that their transmitter content is low. The lowest transmitter concentration is usually found in the dendrites of a neuron, which in this case then would imply that these 5-hydroxytryptamine accumulating bipolar-like cells transmit centrifugally, a condition not associated with bipolar cells. The only cell type in the retina having this signalling direction are interplexiform cells, but more evidence is necessary before it can be suggested that the bipolar-like cells are a morphological variant of such cells.

3.1.2 Indoleamine accumulating neurons in the mammalian retina (I, II, and III)

3.1.2.1 Cat, Rabbit: In both these species, indoleamine accumulating neurons have previously been described in formaldehyde histofluorescence studies (Ehinger and Florén, 1976). In the cat, intraocular injections of indoleamines resulted in an increase in the number of fluorescent perikarya in the innermost part of the inner nuclear layer. There was also a diffuse distribution of fluorescence throughout the inner plexiform layer in a manner that was totally different from the normally occurring dopamine fluorescence, which was confined to a single sublayer of fluorescent terminals at the border between the inner nuclear and inner plexiform layers.

In the normal rabbit retina, dopaminergic cell bodies were found in the innermost cell row of the inner nuclear layer with their terminals forming three sublayers in the inner plexiform layer, the outermost being the best developed. After intravitreal injection of an indoleamine, additional

perikarya appeared and the innermost sublayer became the best developed. Autoradiography after injection of (^3H)-5-hydroxytryptamine revealed the same picture (Ehinger and Florén, 1978; Osborne et al., 1982a). However, no 5-hydroxytryptamine immunoreactive neurons could be detected in either species. This suggests a low 5-hydroxytryptamine content which was corroborated by the quantitative analyses further discussed below.

3.1.2.2. Cow, Pig: The normal retina from cow and pig contained neurons with greenish fluorescence. Rarely occurring, rounded or ovoid cell bodies were found in the innermost third of the inner nuclear layer, sending processes to a single nerve fibre layer situated in sublamina 1 of the inner plexiform layer. A few fibres could also infrequently be seen in the middle and innermost parts of this layer. Incubation in alpha-methylnoradrenalin (1 or 10 $\mu\text{g/ml}$) resulted in increased fluorescence intensity but no additional neurons occurred. If the incubation was performed in 5,6-dihydroxytryptamine, the above described structures turned yellowish but no additional neurons appeared. If very high doses (30 or 50 $\mu\text{g/ml}$) were used, diffuse fluorescence appeared in the innermost layers in the retina and in pericytes around small vessels. Fluorescence also appeared in bipolar-like cells situated in the middle of the inner nuclear layer which sent processes both outwards to the outer plexiform layer and inwards to the inner plexiform layer. They resembled the bipolar-like cells seen in pigeon and chicken, but the significance of this uptake was doubtful, as the doses of 5,6-dihydroxytryptamine needed to demonstrate them also produced nonspecific fluorescence in non-neuronal elements. Thus no indoleamine accumulating neurons could be found with the Falck and Hillarp technique. However, if auto-

radiography was performed after incubation with (^3H)-5-hydroxytryptamine, radioactivity appeared in two sublayers situated in sublaminae 1 and 3 of the inner plexiform layer. In the cow retina, the outermost sublayer was usually the best developed, whereas in the pig retina, the innermost was usually the most prominent. In the cow retina, radioactive cell bodies were found in the innermost two cell rows of the inner nuclear layer and, rarely, also in the middle of the inner plexiform layer. In the pig retina, radioactive cell bodies were frequent in the innermost two or three cell rows of the inner nuclear layer. They were often seen to send coarse radioactive processes into the inner plexiform layer, participating in forming both sublayers. Radioactive cell bodies were occasionally seen in the middle of the inner plexiform layer and in the ganglion cell layer. The latter sent processes to either of the sublayers in the inner plexiform layer. This ability to accumulate (^3H)-5-hydroxytryptamine in the bovine retina has also been observed by others (Thomas and Redburn, 1979; 1980; Osborne, 1980; Osborne and Richardson, 1980; Osborne et al., 1982a). However, no endogenous 5-hydroxytryptamine was found with immunohistochemistry in these retinae. The results agree well with those obtained with the quantitative analyses.

3.1.2.3 Man, Baboon, Cynomolgus Monkey, Guinea-Pig, Rat, Mouse: Immunohistochemistry revealed no endogenous 5-hydroxytryptamine in any of these species. Furthermore, no indoleamine accumulating neurons have been demonstrated with formaldehyde fluorescence histochemistry and/or autoradiography in any of the species. (Florén, 1978; Ehinger and Florén, 1979; papers II and III). However, mouse has not been investigated with these latter methods.

The findings of indoleamine accumulating neurons in the retina are summarized in Fig. 2.

	FLUORESCENCE										AUTORADIOGRAPHY	
	Lamprey	Necturus	Goldfish	Frog	Pigeon Chicken	Rabbit	Cat	Rat Guinea- pig?	Cyna- molgus Homo	Cebus	Cow	Pig
Ph												
ONL												
OPL												
INL												
IPL								absent?	absent?			
G												

Fig. 2. Schematic drawing of indoleamine accumulating neurons in the retina. 5-hydroxytryptamine immunoreactive neurons were found in goldfish, frog, chicken and pigeon (lamprey and necturus not investigated), whereas in mammals no endogenous 5-hydroxytryptamine was found by immunohistochemistry. In cow and pig, indoleamine accumulating neurons were found with autoradiography only. Ph, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer.

3.1.3 Quantitative analyses of 5-hydroxytryptamine in the retina (II, III)

The results of the quantitative analyses are listed in Table 3.

TABLE 3

Quantitative analyses of 5-hydroxytryptamine in the retina

Species	No. of analyses	ng/g ₊ wet weight retina mean-s.e.m
Cow	16	26.2 [±] 7.17
Pig	16	5.9 [±] 0.83
Normal rabbit (2 retinae per analysis)	17	24 [±] 5*
Perfused rabbit (2 retinae per analysis)	19	3.8 [±] 0.9
Guinea-Pig (adult, 5 retinae per analysis)	6	6.03 [±] 2.61
Pigeon (adult)	12	84.8 [±] 10.7
Chicken (6-8 weeks)	8	121.2 [±] 7.02
Frog (adult, 5 retinae per analysis)	4	1720 [±] 420
Goldfish (10-15 cm length, 5 retinae per analysis)	6	1111 [±] 146.4

*From Florén and Hansson, 1980

The highest concentrations of 5-hydroxytryptamine were found in retinae from cold-blooded animals and these figures can be compared with those reported by others. In frog retinae, Welsh (1964) found as much as up to 800 ng/g wet

weight and Osborne (1982b) and Osborne et al. (1981a, 1982a) noted about 500 ng/g wet weight. In frog as well as in goldfish retinae, where Osborne et al. (1982a) reported a 5-hydroxytryptamine content of about 230 ng/g, our figures were higher. The reason for this is unclear but certainly not technical as values in the expected range were obtained from simultaneously processed rabbit retinae. The variability was rather wide in all analyses of frog and goldfish. This is indicated by the high s.e.m. found in these species and suggests considerable individual variations. Alimentary factors which are hard to control may contribute to such variations (Gibson et al., 1982).

In the bird retina, lower amounts of 5-hydroxytryptamine were found than in the cold-blooded animals. Those for the chicken retina were in good agreement with the reports of Suzuki et al. (1977), Parkinson and Rando (1981), and Osborne (1982a). The lowest concentrations were found in the mammalian retina and our figures fall within the same range as those reported by others in the rabbit retina (Florén and Hansson, 1980; Osborne, 1980; Osborne et al., 1982a) and in the cow retina (Osborne 1980, Osborne 1982b, Osborne et al., 1982a). Thomas and Redburn (1979) reported considerably higher values, about 100 ng/g, in the bovine retina, using a fluorimetric method which is less sensitive and presumably less specific than a HPLC-system. Other indoleamines than 5-hydroxytryptamine may therefore have compromised their figures. Moreover, the level of detectability in the fluorimetric method was 40 ng and blank errors may then play a considerable role when measuring total amounts of 1-50 ng, as is the case when analyzing 100-500 mg retina with a concentration in the order of 10-100 ng/g wet weight.

3.1.4 Aspects on 5-hydroxytryptamine as a retinal neurotransmitter

For the identification of a chemical substance as a neurotransmitter there are four main criteria:

1. The substance must be synthesized and/or stored within the neurons, particularly in their presynaptic terminal processes.
2. There must be mechanisms for the destruction or removal of the proposed transmitter at the synaptic site.
3. Nerve activity must lead to release of the substance from nerve terminals.
4. The actions of the substance on the target organ must mimic the effect of nerve activity.

In birds and cold-blooded animals, the first three criteria can be regarded as fulfilled, considering :

- 1) the high amounts of 5-hydroxytryptamine found by HPLC and the presence of endogenous 5-hydroxytryptamine as demonstrated by immunohistochemistry (Osborne et al., 1981a; Parkinson and Rando, 1981; Osborne, 1982a,b; Osborne et al., 1982a; paper III). Furthermore, 5-hydroxytryptamine immunoreactivity in the frog retina is markedly decreased by prior treatment with reserpine (which interferes with storage of transmitters) or p-chlorophenylalanine, an inhibitor of tryptophan hydroxylase which is the rate-limiting enzyme in the synthesis of 5-hydroxytryptamine (Osborne et al., 1982a). Intraocular injection of reserpine has also been shown to cause a significant decrease in the 5-hydroxytryptamine content in the chicken retina (Parkinson and Rando, 1981).

- 2) 5-hydroxytryptamine is readily taken up in neuronal elements as shown with autoradiography or formaldehyde histofluorescence (Ehinger and Florén, 1976; Florén, 1979a; Osborne et al., 1982a; Osborne, 1982a; Ehinger, unpublished observations; paper VII). In the chicken retina, the uptake system has been found to consist of two saturable uptake mechanisms with different K_m s. The uptake is temperature sensitive, sodium dependent, and strongly diminished by inhibitors of 5-hydroxytryptamine uptake such as Lilly 110140 or chlorimipramine (Suzuki et al., 1978; Osborne, 1982a). The high affinity uptake is thought to constitute the neuronal re-uptake of transmitter released from presynaptic terminals (Iversen, 1971), whereas low affinity uptake is thought to be due to unspecific processes such as passive accumulation, unspecific binding or incorporation into tissue (e.g. Iversen 1971). A high affinity uptake process has been registered also in the frog retina (Osborne et al., 1981a).
- 3) The accumulated 5-hydroxytryptamine can be released by K^+ in a Ca^{2+} dependent manner which is typical for neuronal release (Osborne et al., 1981a; Osborne, 1982a). However, to date there are no reliable data on light-evoked release of either radioactive or endogenous 5-hydroxytryptamine in these species.
- 4) The fourth criterion is the so called demonstration of identity of action. Indirect studies on this include analysis of receptor binding and postsynaptic response of cyclic AMP to presynaptic release of neurotransmitter or external application of the substance. No data are avail-

able on this in birds or cold-blooded animals. Nevertheless, the other three criteria provide good support for 5-hydroxytryptamine being the neurotransmitter of the indoleamine accumulating neurons in birds and cold-blooded animals.

In the mammalian retina the results are less consistent. The 5-hydroxytryptamine concentrations are low, and in the rabbit retina the concentration can be significantly decreased by eliminating the thrombocytes, indicating that much of the measured 5-hydroxytryptamine is localized within them. If 5-hydroxytryptamine is present in neurons at all, it is below the level of detectability of the very sensitive immunohistochemical method. This is not typical for well proven 5-hydroxytryptamine neurons. Further, enzymes capable of metabolizing 5-hydroxytryptamine are present in the retina (Osborne, 1980; Ehinger, unpublished observations) but this activity appears to be low because Florén and Hansson (1980) were unable to quantify tryptophan hydroxylase in the rabbit retina.

There is no doubt an uptake mechanism for 5-hydroxytryptamine in cow, pig, cat or rabbit as shown with autoradiography, formaldehyde histofluorescence, and kinetic uptake studies (Ehinger and Florén, 1976; Ehinger and Florén, 1978; Thomas and Redburn, 1979; 1980; Osborne 1980, Osborne and Richardson, 1980; Osborne 1981; Redburn and Thomas, 1981; Osborne et al., 1982a). The uptake mechanism has by several authors been found to be a high-affinity uptake system which is temperature sensitive, sodium dependent, saturable and inhibited by drugs known to influence amine uptake such as chlorimipramine and Lilly 100140 (Ehinger and Florén, 1978;

Osborne, 1980; Thomas and Redburn, 1979, 1980). The accumulated (^3H)-5-hydroxytryptamine in the bovine retina can then be released by K^+ in a Ca^{2+} dependent manner (Thomas and Redburn 1979, 1980; Osborne 1980). However, the release produced by flashing light or by K^+ in the rabbit retina was not comparable to that found with other transmitters such as glycine or dopamine (Bauer et al., 1983). The discrepancy in results between formaldehyde histofluorescence and autoradiography may indicate that the 5-hydroxytryptamine taken up by the retina is metabolized into a non-fluorogenic form which is then released upon stimulation. The identity of this metabolite is still unknown. Melatonin and bufotenine are not fluorogenic in the method of Falck and Hillarp, but are unlikely candidates as they are not taken up by neurons in the retina and have little effect on the indoleamine uptake system (Ehinger and Florén, 1978). The antiserum used for the immunohistochemical demonstration of 5-hydroxytryptamine was found to cross-react with 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, bufotenine and 6-hydroxytryptamine. None of these are from other studies known to be present in the retina. Therefore, the immunofluorescence is not likely to be caused by any of these substances. Osborne (1982b) has hypothesized that 5-hydroxytryptamine is a transmitter also in the mammalian retina, explaining the low content of the putative transmitter with the speculation that neurons using a specific transmitter become more efficient and thereby need a lower transmitter level as animals progress higher up the evolutionary scale. It is on the other hand equally possible that neurons during evolution may change their transmitter, i. e. if two transmitters co-occur within one neuron it may during one period use one of the two substances as its transmitter, during another period perhaps both and, eventually only the

other transmitter. Such neurons would still most likely possess the ability to accumulate the abandoned transmitter. It is at present not possible to decide between the two alternatives, nor can more complicated explanations be excluded. More and consistent data are needed before 5-hydroxytryptamine can be established as a transmitter in the retina of mammals.

3.2 PEPTIDE CONTAINING NEURONS IN THE RETINA

3.2.1 Somatostatin (IV, V)

Somatostatin containing neurons have been identified in a number of different species. Cell bodies were rare and impossible to identify in many species but have been seen in squirrel monkey, pig, pigeon, chicken, frog and goldfish. When cell bodies were found, they were ovoid, rounded or pear-shaped, and invariably localized among the amacrine cells in the innermost cell rows in the inner nuclear layer. Fine, varicose nerve fibres generally formed two or three sublayers in sublaminae 1, 3 and 5 of the inner plexiform layer. In some species, vertically running nerve fibres were seen to connect them. In birds and frogs, the pattern of ramification was slightly different. In the bird retina, a single sublayer was seen in sublamina 1, whereas a loosely tiered plexus of nerve fibres was seen throughout sublaminae 3 and 4. In the frog, nerve fibres formed a loose plexus in the outermost third of the inner plexiform layer and vertically running nerve fibres connected this plexus with a single sublayer in sublamina 5. In cow, pig, cat, rat and frog, immunoreactive fibres were also found at the border between the inner nuclear and outer plexiform layers. In some species, they were clearly seen to connect the inner and outer plexiform layers in the way interplexiform neurons do. They have consequently been regarded as interplexiform cells, though information on their synaptic

contacts and also on their physiology is still lacking. The findings on somatostatin immunoreactive neurons are summarized in Fig. 3. In addition, a few somatostatin fibres have been noted at the border between the inner nuclear and inner plexiform layers in the human retina (Tornqvist, unpublished observations).

The findings were in good agreement with those presented by others in the chicken and in the rat retina (Buckerfield et al., 1981; Eriksen and Larsson, 1981; Fukuda, 1982). The distribution of neurons and the pattern of stratification in the chicken retina was also very similar to that found in pigeon (Brecha et al., 1981a). However, in a few respects the

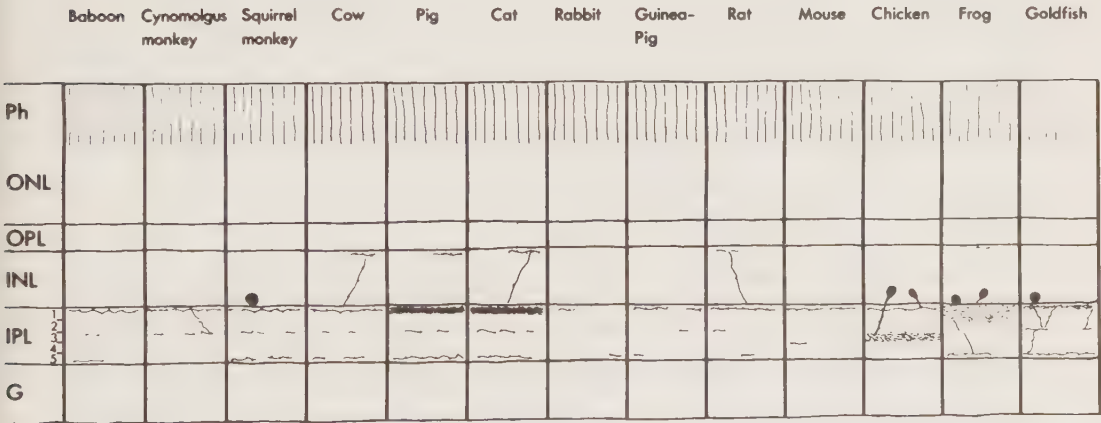


Fig. 3. Schematic drawing illustrating the morphology of somatostatin immunoreactive neurons in all species investigated except man. Fibres usually formed two or three more or less well defined sublayers in the inner plexiform layer. Cell bodies were ovoid or slightly pear-shaped and situated in the innermost part of the inner nuclear layer. Interplexiform somatostatin immunoreactive fibres were observed in cow, pig, cat, rat and frog. Designation of layers as in Fig. 2.

findings in this study were dissimilar to those from other laboratories. Krisch and Leonhardt (1979) reported somatostatin immunofluorescent perikarya and nerve fibres at three locations in the rat retina: 1) in the ganglion cell layer 2) at the border between the inner nuclear and inner plexiform layers and 3) at the border between the inner nuclear and outer plexiform layers. These results were obtained with one particular antibody, whereas another antibody produced no positive results. This indicates that the dissimilarities are due to differences in specificity or potency of the antibodies used. In the goldfish retina, Yamada et al. (1980) have reported the occurrence of somatostatin immunoreactive ganglion cells as well as amacrine cells. No ganglion cells were seen in the present work, but since they were noted as scarce in the report by Yamada et al. (1980) the different results may be due to random variations.

Interplexiform cells containing somatostatin have previously not been described.

The immunohistochemical findings of somatostatin immunoreactive material in the retina have been confirmed and supplemented by radioimmunoassay or bioassay performed on extracts from retinæ from the following species: man (Rorstad et al. 1980), monkey (Macaca Mulatta; Eskay et al. 1980), cow (Eskay et al. 1980, Yamada et al. 1980), rabbit (Eskay et al. 1980, Unger et al. 1981), guinea-pig (Eskay et al. 1980), rat (Rorstad et al. 1979, Shapiro et al. 1979, Eskay et al. 1980, Lake and Patel 1980), pigeon (Brecha et al. 1981a), chicken (Eskay et al. 1980) bullfrog (Eskay et al. 1980), frog and goldfish (Yamada et al. 1980).

3.2.2 VIP (vasoactive intestinal polypeptide (V, VIII))

Investigation of different species has shown that VIP neurons are less ubiquitous than the somatostatin ones. However, they have been found in a considerable number of mammals (paper V) and birds (Fukuda et al., 1981a; Karten and Brecha, 1982; Fukuda, 1982; Brecha and Karten, 1983), although not in cold-blooded animals. In the retina of all species except baboon and cynomolgus monkey, cell bodies were localized among the amacrine cells in the innermost cell row of the inner nuclear layer with nerve fibres forming two or three sublayers in sublaminae 1, 3 and 5 of the inner plexiform layer. In the cynomolgus monkey, nerve fibres were confined to a single sublayer in sublamina 3, whereas no cell bodies were found. In the baboon retina, both cell bodies and nerve fibres were found only in sublamina 3 of the inner plexiform layer as shown in Fig. 4. This localization of neurons is also known from metal impregnation studies (Ramon y Cajal in Rodieck, 1973). Such cells have traditionally been regarded as displaced amacrine cells. However, the observation that they are the only cells containing a specific neuropeptide, VIP, suggests that they have their peculiar localization because of some functional requirement rather than by accident and the term interstitial amacrine cells is therefore preferable. Recently, interstitial amacrine cells displaying substance P immunoreactivity have been described in the retina of a New World monkey, the squirrel monkey (Brecha et al., 1982) and such substance P immunoreactive cells have also been noted in the human retina (Tornqvist, unpublished observations). In chicken retina, VIP immunoreactive cell bodies have been noted both in the inner nuclear and inner plexiform layers (Fukuda, 1982).

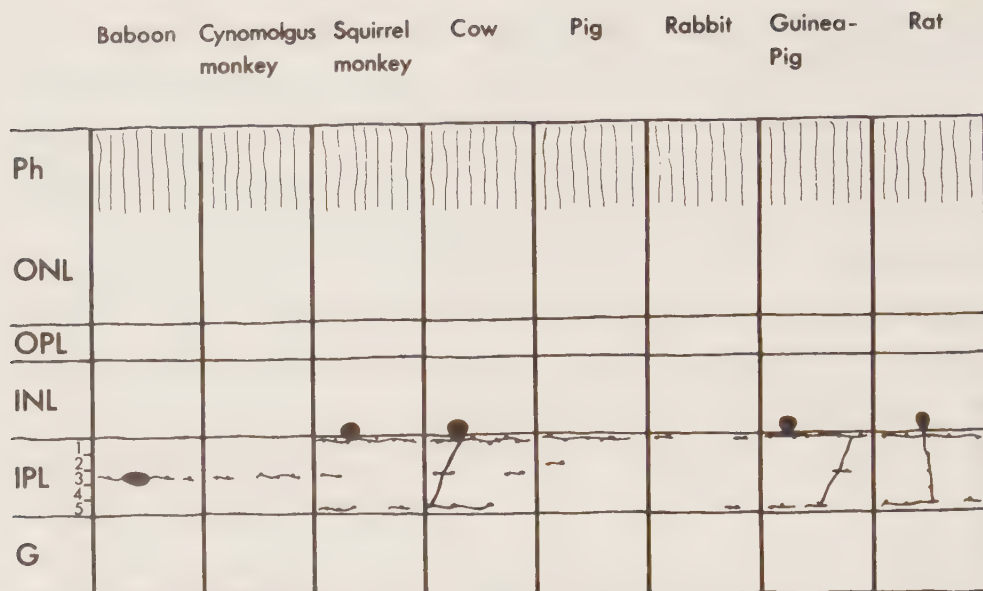


Fig. 4. Schematic drawing illustrating the results of the morphological study on VIP immunoreactive neurons. The findings in man are not shown in Fig. The VIP fibres were coarser than the somatostatin fibres. The VIP cell bodies also occurred more frequently and were usually somewhat larger than the somatostatin ones. In the baboon retina VIP neurons were found exclusively in the middle of the inner plexiform layer constituting interstitial amacrine cells. Designation of layers as in Fig. 2.

In some species, the localization and the distribution of VIP neurons may superficially resemble that of the somatostatin ones. However there are clear differences. The VIP cell bodies are much more frequent than the somatostatin ones, and they are usually somewhat larger, rounded or at times polyhedral instead of ovoid or pear-shaped. The VIP fibres are generally coarser than the somatostatin ones. Moreover, they are differently distributed in baboon, cynomolgus monkey and bird retina. Finally, no VIP immunoreactive interplexiform

fibres have been identified. The findings on VIP immunoreactive neurons in the retina are summarized in Fig. 4.

VIP immunoreactive neurons have been demonstrated in the rat retina also by Eriksen and Larsson (1981) and their results are in good agreement with ours. In addition, they reported a considerably increased immunofluorescence in light-adapted rats compared to dark-adapted animals. VIP neurons have also been demonstrated in retinae from pigeon (Karten and Brecha, 1982; Brecha and Karten, 1983) and chicken (Fukuda et al., 1981a). In both species, as in mammals, the cell bodies were found in the innermost cell rows in the inner nuclear layer, but their processes ramified in a different manner. In the pigeon retina processes were present within sublaminae (1), 3 and 5 of the inner plexiform layer, whereas in the chicken retina, processes ramified into sublayers in sublaminae 1 and 4. In the human retina, scarce VIP immunoreactive, pear-shaped cell bodies with processes ramifying in the outermost part (sublaminae 1-2) of the inner plexiform layer have been observed (Tornqvist, unpublished observations).

To confirm the immunohistochemical findings, radioimmunoassay of VIP was performed in a number of species as listed in Table 4.

TABLE 4

Concentration of VIP-like immunoreactive material in the
retina

species	number of analyses	VIP-concentration* pg/mg retina (wet weight) mean $\pm$ S.E.M.
Cow	6	25.4 $\pm$ 3.6
Pig	8	15.3 $\pm$ 3.9
Cat	8	0.9 $\pm$ 0.4
Rabbit	9	11.6 $\pm$ 3.4
Guinea-Pig	8	0.3 $\pm$ 0.1
Rat	8	72.4 $\pm$ 15.3
Goldfish	4	0.8 $\pm$ 0.5

* expressed as equivalents of porcine VIP

The results were, with one exception, in good agreement with those obtained with immunohistochemistry. No VIP immunoreactivity was observed in retinae from cat or goldfish, and in these species the concentrations of VIP-like material were low. In the guinea-pig retina, very low concentrations of VIP immunoreactive material were found though VIP neurons could readily be demonstrated by immunohistochemistry. The cause of this discrepancy remains obscure but is not likely to be due to technical problems, as radioimmunoassay on extracts from guinea-pig retina performed at different times were consistent, and since results within the expected range were always obtained in extracts from other species processed simultaneously. Furthermore, all extracts diluted parallel to the standard curve in serial dilutions indicating that there is no reason to presume any difference between the antigen in the extract and the standard.

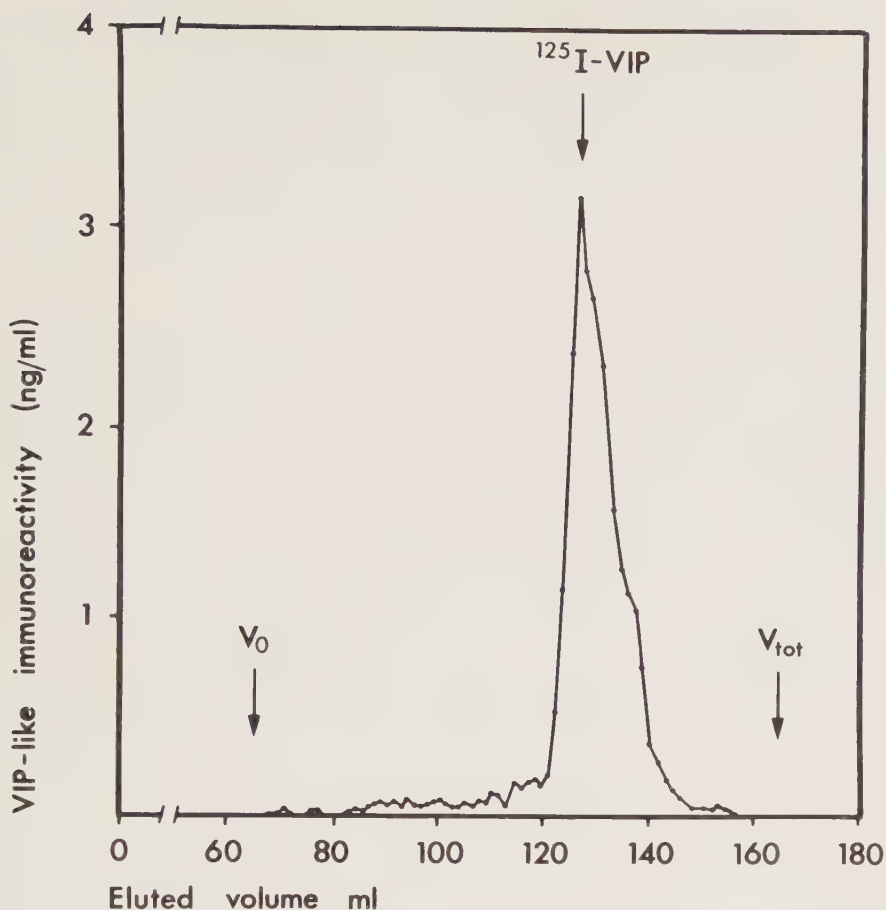


Fig. 5. Gel chromatography of VIP immunoreactive material extracted from 2.3 g bovine retina. Chromatography was performed on a Sephadex^(R) G-50 superfine column. The column was calibrated with Blue Dextran, (^{125}I)-labelled VIP and (^3H)-glycine. Arrows indicate void volume (V_0), total volume (V_{tot}) and the elution position of porcine (^{125}I)-labelled VIP.

The VIP-like material in the retina was further characterized by gel chromatography of extracts (n=7) from bovine retinae. As shown in Fig. 5, the extracted protein had the same elution position as (^{125}I)-labelled VIP. There can thus be no or only minor differences between the standard and the extracted material.

3.2.3 Glucagon (IV, VI, VIII)

Glucagon immunoreactive neurons in the retina seem to be restricted to birds and cold-blooded animals (papers IV, VI, VIII; Fukuda, 1982; Kuwayama et al. 1982; Brecha and Karten, 1983). Though immunoreactivity has been searched for in all species displaying somatostatin immunoreactive neurons (Fig. 3) including pigeon and man, glucagon immunoreactive neurons have only been identified in retinae from pigeon, chicken, frog and goldfish. In the pigeon retina two types of cell bodies were found. One was more frequent, rounded or slightly polyhedral and about the same size as the surrounding non-fluorescent perikarya. These cells were situated in the innermost cell row of the inner nuclear layer and sent processes to a continuous sublayer in sublamina 1 as well as to a discontinuous one in sublamina 3. They were occasionally seen to be connected by radially running nerve fibres. The other cell type was strongly fluorescent, more elongated and about three times larger than the first type. Its axon hillock was readily visible and its processes ramified in sublaminae 1, 3 and 5. These cells were reported also by Brecha and Karten (1983). Their processes seem to join a circular bundle of glucagon immunoreactive processes at the ora serrata.

In the chicken retina, neurons similar to those first described in pigeon retina (see above) were observed. The cell bodies were rounded or slightly polyhedral, situated in the innermost cell row of the inner nuclear layer and with processes ramifying in sublaminae 1 and 3 of the inner plexiform layer. Others (Fukuda, 1982; Kuwayama et al., 1982) have obtained similar results and Kuwayama et al. (1982) also demonstrated that glucagon immunoreactive neurons seem to be evenly distributed in the retina. They observed two cell types, one smaller (7 μm) and one somewhat larger (10 μm). The latter seemed to be located preferentially in the peripheral retina. This cell type has not with certainty been detected in this study but it may have escaped detection, as mainly central parts of the retina were used.

In the frog retina, glucagon immunoreactive neurons were more frequent than in pigeon or chicken. Cell bodies were rounded or slightly polyhedral and with the same location as in the bird retina. Nerve fibres formed a loose plexus in the outermost third of the inner plexiform layer. At times two distinct sublayers with radial communications between them could be discerned within this plexus. Radial nerve fibres projected inwards, reaching a single sublayer of immunoreactive nerve fibres in sublamina 5. Immunoreactive cells were also occasionally found in the ganglion cell layer. Such cells sent processes to both sublayers previously described. They were, on the other hand, never seen to send nerve fibres to the optic nerve and thus seemed to constitute displaced amacrine cells rather than ganglion cells.

In the goldfish retina, cell bodies were sparse, rounded or slightly polyhedral, and were situated in the innermost cell row in the inner nuclear layer. Nerve fibres formed a single sublayer in sublamina 1. Scattered varicose fibres were seen in sublamina 3 and, rarely, nerve fibres were also seen to project from sublamina 1 to the fibres seen in sublamina 3.

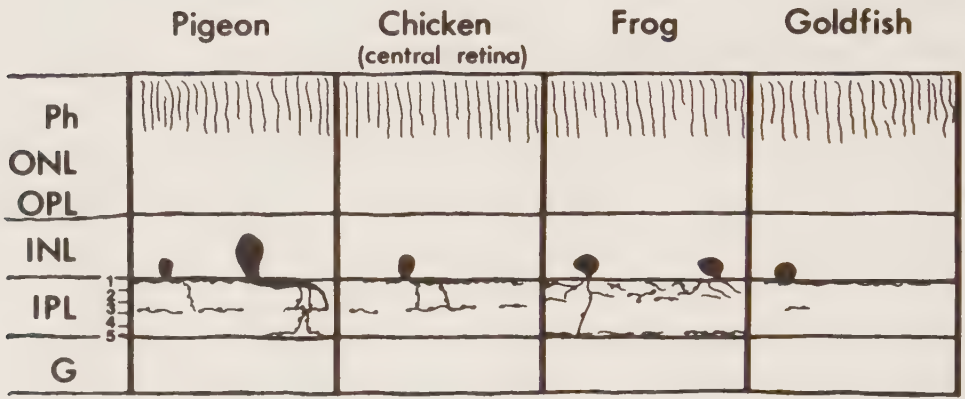


Fig. 6. Schematic drawing illustrating the morphology of the glucagon immunoreactive neurons described. In the pigeon retina, two different types of cell bodies with different patterns of stratification were observed. In birds and goldfish, the neurons were strictly stratified. In the frog retina, a loose plexus of nerve fibres was found in the outermost part of the inner plexiform layer. A single nerve fibre layer was seen in sublamina 5. The rarely occurring immunoreactive cell bodies observed in the ganglion cell layer in the frog are not shown in Fig. Designation of layers as in Fig. 2.

The findings on glucagon immunoreactive neurons are illustrated in Fig. 6.

To confirm the immunohistochemical results, radioimmunoassay was performed on extracts from retinae from several species.

The concentrations of glucagon immunoreactive material obtained with radioimmunoassay turned out to be in very good agreement with the immunohistochemical observations described above. The highest concentrations were found in the frog retina, where the number of neurons is larger in comparison with, e.g., the bird retina. The lowest concentrations were found in goldfish, where cell bodies and nerve fibres are scarce. In mammals, the amounts were found to be very small or even undetectable. The results are shown in Table 5.

TABLE 5

Concentration of glucagon immunoreactive material in the
retina

Species	number of analyses	Glucagon concentration* pg/mg retina (wet weight) mean $\pm$ S.E.M.
Cow	4	0.2 $\pm$ 0.01
Pig	4	0.4 $\pm$ 0.2
Rabbit	6	0.04 $\pm$ 0.04
Rat	2	not detectable
Pigeon	4	19.5 $\pm$ 9.4
Chicken	10	14.4 $\pm$ 1.2
Frog		
(<i>Rana temporaria</i>)	7	35.5 $\pm$ 4.0
Goldfish		
(<i>Carassius auratus</i>)	6	9.2 $\pm$ 1.2

* expressed as equivalents of porcine pancreatic glucagon

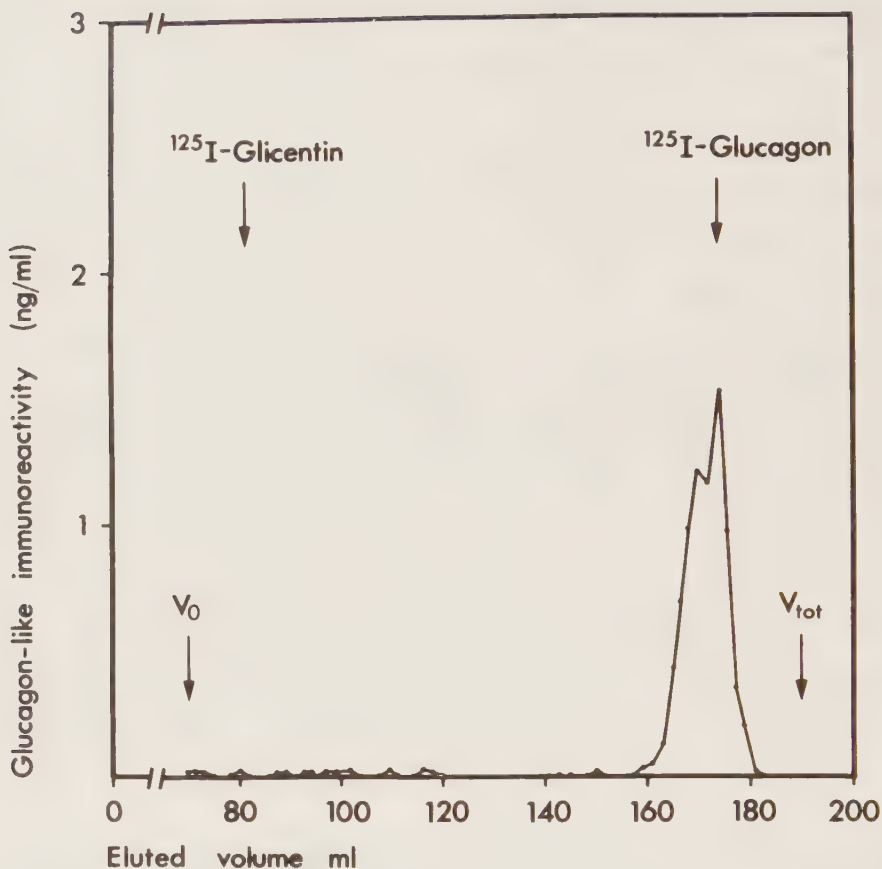


Fig. 7. Gel chromatography of glucagon immunoreactive material extracted from 0.4 g frog retina. Chromatography was performed on a Sephadex^(R) G-50 superfine column. The column was calibrated with Blue Dextran (^{125}I)-labelled porcine glicentin, (^{125}I)-labelled porcine glucagon and (^{125}I). Arrows indicate void volume (V_0), total volume (V_{tot}) and elution positions of glicentin and glucagon respectively. The elution position of immunoreactive material extracted from chicken retina was identical with that extracted from frog retina (not shown in Fig.)

Extracts from chicken and frog retinæ were chromatographed in order to obtain further characteristics of the glucagon immunoreactive material. Both gel chromatography and HPLC were used. The antiserum used for immunohistochemistry as well as for radioimmunoassay is known to cross-react with glicentin (gut-type glucagon) and this substance was consequently used as reference standard in addition to glucagon. As shown in Figs. 7 and 8, the extracted protein from frog and chicken retinæ in both systems had the same elution position as (^{125}I)-labelled or pure porcine pancreatic-type glucagon. No immunoreactive material had the elution position of glicentin. The results strongly suggest that the immunoreactive material demonstrated with immunohistochemistry is glucagon.

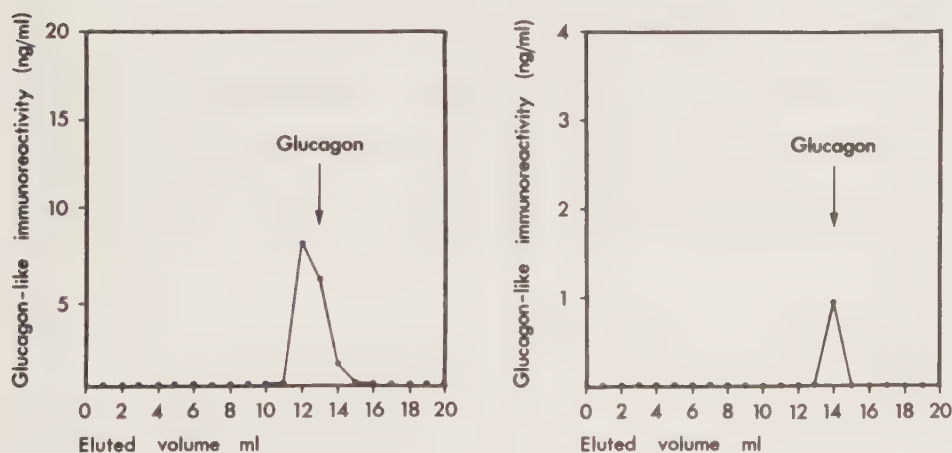


Fig. 8. HPLC profile of glucagon-like immunoreactivity in extracts from 0.84 g chicken retina (left) and 0.56 g frog retina (right). HPLC was performed on a μ -Bondapack C₁₈ column with acetonitrile/0.1 M phosphate buffer as eluant. A linear gradient from 32-50% CH₃CN was applied. There was good agreement between the elution positions of the extracted glucagon-like material and pure porcine glucagon (arrow).

3.2.4 Enkephalin (IV)

Enkephalin immunoreactive neurons have been described in retinae from pigeon (Brecha et al., 1979) and chicken (paper IV; Fukuda, 1982). Such neurons have in two review papers also been mentioned in goldfish and some other cold-blooded animals (Stell et al., 1980; Brecha and Karten, 1983).

In pigeon and chicken, the number and distribution of cell bodies and nerve fibres was found to be very similar (Brecha et al., 1979, paper IV). Numerous ovoid or pear-shaped cell bodies were situated in the innermost cell rows of the inner nuclear layer. They sent processes to a single sublayer in sublamina 1 as well as to a broad and much less well defined layer in sublaminae 3 and 4. The two sublayers were connected by radially running fibres.

The presence of enkephalin immunoreactivity in the chicken retina has been confirmed by radioimmunoassay whereby Humbert et al. (1979) found both met-enkephalin and leu-enkephalin immunoreactive material.

Despite extensive search, no enkephalin immunoreactive neurons were found in the retina of any mammal. However, very recently, Altschuler et al. (1982) described enkephalin immunoreactive neurons in the retina of guinea-pigs with the aid of a special antiserum. The cell bodies were located in the innermost cell row of the inner nuclear layer and their processes ramified in sublaminae 1, 3 and 5 of the inner plexiform layer. The pattern of stratification thus differs entirely from that seen in birds. The observations with this particular antiserum have been confirmed (Ehinger and Tornqvist, unpub-

lished observations), showing that different antisera can give contrasting results. The exact chemical identity of the enkephalin immunoreactive material in the guinea-pig retina remains unknown.

In the goldfish retina, the enkephalin containing amacrine cells were reported as bistratified or multistratified (Stell et al., 1980) but no enkephalin neurons were seen in this animal in the present study. The reason for this may well be differences in the antisera, as exemplified by the results in guinea-pig discussed above.

3.2.5 Neurotensin (IV)

Neurotensin immunoreactive neurons have so far been demonstrated in birds only (paper IV; Brecha et al., 1981a). Unlike the case with somatostatin and enkephalin immunoreactive neurons, differences are noted when comparing pigeon and chicken. In the pigeon retina (Brecha et al., 1981a), the neurons were found to be numerous, ovoid, and situated in the innermost cell rows of the inner nuclear layer. The processes ramified in a diffuse plexus in sublamina 1 which by radially running nerve fibres was connected with a dense plexus of nerve fibres situated in sublaminae 3 and 4. In the chicken retina (paper IV), the ovoid or pear-shaped cell bodies had about the same localization as in pigeon. However, nerve fibres were not seen to form plexa like those in the pigeon, but only two, weakly fluorescent nerve fibre layers in sublaminae 1 and 3. This difference is certainly not due to differences in antisera used, as the same antibody (HC 8) was used as in the study by Brecha et al. (1981a). The observations were also repeated with a different antibody (code no. 47189). Fukuda (1982) recently reported similar,

though not entirely identical, results in chicken. In addition to neurotensin immunoreactive amacrine cells, he also observed immunoreactive ganglion cells. Again, the immunohistochemical findings are strengthened by the results of radioimmunoassay (Brecha et al., 1981a).

There is thus a considerable similarity in the morphology of somatostatin, enkephalin, and neurotensin immunoreactive neurons in the pigeon retina and also to a certain extent in the chicken retina. The question whether these substances co-occur in the same type type of cell or whether the neurons constitute three separate cell types will be discussed below.

3.2.6 Substance P, cholecystokinin, TRH, LH-RH

The morphology of neurons containing substance P, cholecystokinin, TRH and LH-RH in different species will briefly be described below to facilitate the discussion on the possible co-occurrence of two peptides within one neuron.

Substance P has been described in retinal neurons in monkey, rabbit, rat, pigeon, chicken, mudpuppy, lizard, frog and goldfish (Karten and Brecha, 1980; Brecha et al., 1981b; Eskay et al., 1981; Fukuda et al., 1981b; Osborne et al., 1982b; Brecha et al., 1982; Fukuda, 1982; Ishimoto et al., 1982; Brecha and Karten, 1983). In the rabbit retina, two types of substance P immunoreactive cell bodies were found at the border between the inner nuclear and inner plexiform layers. One cell type gave rise to a stout process which ramified in sublamina 5 of the inner plexiform layer, whereas processes from the other type ramified in sublaminae 1, 3 and 5. Substance P immunoreactive cell bodies were also found among the ganglion cells. The processes from these ramified in

sublamina 5. A few fibres were also found in the outer plexiform layer (Brecha and Karten, 1983). In the rat retina, cell bodies in the innermost part of the inner nuclear layer gave rise to two immunoreactive nerve fibre layers in the innermost and outermost parts of the inner plexiform layer (Fukuda et al., 1981b; Osborne et al., 1982b). In pigeon, chicken, lizard and goldfish, cell bodies were ovoid, situated in the innermost part of the inner nuclear layer, and gave rise to a process that ramified in a single sublayer in Cajal's sublamina 3 (Karten and Brecha, 1980; Brecha et al., 1981b; Karten and Brecha, 1982; Osborne et al. 1982b, paper VII). In the frog (*Rana pipiens*), numerous cell bodies were located in the innermost part of the inner nuclear layer, and their processes ramified widely and diffusely in the inner plexiform layer (Osborne et al., 1982b). Substance P immunoreactive neurons have also been observed in the human retina (Tornqvist, unpublished observations). Ovoid cell bodies were located in the innermost part of the inner nuclear layer and sent stout processes to a continuous nerve fibre layer in sublamina 2 of the inner plexiform layer. At times cell bodies were seen in this nerve fibre layer, and sometimes cell bodies were also noted among the ganglion cells. These cells sent coarse processes outwards to the nerve fibre layer in sublamina 2. Also, nerve fibres were noted in the ganglion cell layer, and additionally at the border between the inner nuclear and inner plexiform layers. Nerve fibres were also seen to run outwards from the inner plexiform layer to a discontinuous nerve fibre layer at the border between the inner nuclear and outer plexiform layers.

Cholecystokinin (CCK) was described in the retina of chicken, frog and lizard (Osborne et al., 1981b; Yamada et al., 1981; Osborne et al., 1982b). In the chicken, processes formed three distinct layers in the outermost, middle, and innermost parts of the inner plexiform layer (Osborne et al., 1982b). In the frog, cell bodies were found in the innermost part of the inner nuclear layer and their processes ramified in the innermost 2/3 of the inner plexiform layer (Yamada et al., 1981; Osborne et al., 1982b).

Thyrotropin releasing hormone (TRH) has been detected in the retina of several different species with radioimmunoassay and its presence in the retina is also suggested by receptor binding studies (Schaeffer et al., 1977; Burt 1979; Youngblood et al., 1979; Martino et al., 1980; Burt and Taylor; 1982). However, no morphological studies are known to date.

Luteinizing hormone releasing hormone (LH-RH) has been described in the retina of paddy-bird and chicken (Fukuda, 1982; Fukuda et al., 1982). In the paddy-bird, immunoreactive cell bodies were found in the inner nuclear, inner plexiform and ganglion cell layers. Nerve fibres formed a single sublayer in sublamina 4. In the chicken retina, cell bodies were found in the innermost part of the inner nuclear layer with nerve fibres distributed in sublaminae 1, 3 and (5) in the inner plexiform layer. In teleostean fish, centrifugally running LH-RH immunoreactive fibres have been reported (Münz et al., 1982). They emanate from a cell group located at the transition of the ventral telencephalon and olfactory bulb and terminate in the contralateral retina at the border between the inner nuclear and inner plexiform layer.

3.3 CO-OCCURRENCE

3.3.1 Co-occurrence of peptides in one neuronal population

Because of the large number of possible transmitter substances in the amacrine cells there is reason to hypothesize that two or more transmitters could occur in one and the same neuron. Such co-occurrence has been described in the brain (Hökfelt et al., 1978; Johansson et al., 1981). When comparing different peptide immunoreactive neuron populations in one and the same species there may be slight similarities, but not in any case are the number of cell bodies, their localization, size and shape, distribution, or pattern of ramification identical. Also, the thickness of the nerve fibres varies. The somatostatin, enkephalin and neurotensin fibres are fine and delicate, whereas VIP and substance P fibres are coarser. The best similarity between different peptide containing neuron populations is found in the pigeon retina where enkephalin, neurotensin and somatostatin immunoreactive neurons are very much alike. However, in double-label experiments, Brecha et al. (1981a) were unable to see any co-occurrence of somatostatin and neurotensin. Even though these neurons are morphologically similar, they are therefore not likely to be identical. There are thus no reasons to believe that any of the neuropeptides detected in the retina co-occur with any other such peptide. However, the precision and sensitivity of these experiments is not such as to exclude the possibility that small nerve cell populations with co-occurrence exist.

3.3.2 Co-occurrence of neuropeptides and conventional neurotransmitters

Several neurotransmitters such as GABA, glycine, dopamine, an indoleamine (see paper III), and acetylcholine occur in amacrine cells (Ehinger, 1982). GABA and glycine neurons

are not well stratified and are therefore not likely to co-occur with any of the peptides described. Neurons containing dopamine and acetylcholine are stratified (Ehinger, 1982) but a careful comparison of the sublayering in the inner plexiform layer does not support the suggestion that these transmitters co-occur with any of the peptides.

3.3.3 Co-occurrence of 5-hydroxytryptamine and peptides in bird retina (VII)

This study was performed with combined autoradiography and immunohistochemistry. The number and distribution of cell bodies and terminals labelled with (^3H)-5-hydroxytryptamine and those shown with immunohistochemistry was in good agreement with previous studies on 5-hydroxytryptamine (Florén, 1979a; Osborne, 1982a; paper III) and the various peptides (Karten and Brecha, 1980; Brecha et al., 1981a; paper IV). There was therefore no reason to believe that any neurons had escaped detection. In one series of sections, 844 somatostatin and 321 (^3H)-5-hydroxytryptamine labelled cells were examined. No double-labelled cells were detected. The same result was obtained when in four different series of sections 173 glucagon immunoreactive and 380 (^3H)-5-hydroxytryptamine labelled cells, 234 substance P immunoreactive and 205 (^3H)-5-hydroxytryptamine labelled, 723 neurotensin immunoreactive and 232 (^3H)-5-hydroxytryptamine labelled, and 147 enkephalin immunoreactive and 113 (^3H)-5-hydroxytryptamine labelled cells were thoroughly examined under high magnification ($> \times 600$). Using statistics based on the binomial distribution, this means that it is possible to state with more than 98% confidence that the true number of double labelled cells was less than 1% of the studied populations.

Thus, to date no co-occurrence of two or more peptides or other transmitters is known in retinal neurons and, consequently, all the different transmitters described in amacrine cells seem to be located in individual neuronal populations.

3.4 POSSIBLE FUNCTIONS OF RETINAL NEUROPEPTIDES

The retinal neuropeptides have been detected only recently, and very little is therefore yet known about their functions. However, VIP has been shown to stimulate the adenylate cyclase in retinae from carp, rat, rabbit, and calf (Longshore and Makman, 1981; Schorderet et al., 1981; Watling and Dowling 1983). This perhaps suggests a relationship with dopaminergic neurons because dopamine is the only other substance known to stimulate the adenylate cyclase of fish horizontal cells (Watling and Dowling, 1981).

Glucagon was found to stimulate the adenylate cyclase in the pigeon but not in the rabbit retina where no glucagon immunoreactive neurons can be demonstrated (Schorderet et al., 1981). Again, this may indicate some connection with dopaminergic neurons.

Substance P and neurotensin have in the amphibian retina been found to have an excitatory effect on ganglion cells, both on ON, OFF, and ON-OFF units (Dick and Miller, 1981). In the carp retina the same excitatory effect of substance P was found mainly on ON-responding ganglion cells (Glickman and Adolph, 1982). This effect of substance P was still present after application of cholinergic blockers and is thus different from that of acetylcholine which is also known to influence these cells. The results suggest the presence of specific SP receptors on ON ganglion cells.

In the goldfish retina, enkephalins were demonstrated to disinhibit ON-type ganglion cells, presumably by inhibiting inhibitory GABAergic amacrine cells (Djamgoz et al., 1981). Very recently Reading (1982) has reported that TRH causes a slow but Ca^{2+} -dependent release of (^3H)-dopamine in dark-adapted bovine retinae. Additionally, TRH blocked light-stimulated but not K^{+} -stimulated release of (^3H)-dopamine. These complex results were suggested to be caused by a modulatory effect of TRH on classical neurotransmitters.

Thus different peptides no doubt have effects in the retina, so that it is possible to surmise that there are receptors for glucagon, VIP, substance P, TRH and enkephalins. Such receptors have also been detected in radioligand studies with the opioid peptides (Borbe, et al., 1982) and TRH (Burt and Taylor, 1982). The present work has demonstrated that they also occur presynaptically in certain neurons in the retina, and this suggests they may have a regulatory function. It is not yet known if the effects are fast enough to regard any of the peptides as conventional, fast neurotransmitters. Also, release of retinal neuropeptides as a result of nerve activity remains to be demonstrated. Nevertheless, the physiological and morphological results obtained so far strongly suggest important roles for the different neuropeptides in the retina.

4. GENERAL SUMMARY

5-hydroxytryptamine immunoreactive neurons were found among the amacrine cells in goldfish, frog, chicken and pigeon. The neurons were strictly stratified in the goldfish, chicken, and pigeon retina whereas nerve fibres ramified throughout the inner plexiform layer in the frog retina. In the bird retina, immunofluorescent cell bodies were also found in the middle of the inner plexiform layer, and their nerve fibres were seen to project both inwards and outwards. These are the so-called bipolar-like cells. High concentrations of 5-hydroxytryptamine were detected in these four species, and the data strongly suggest that 5-hydroxytryptamine is the transmitter of the indoleamine accumulating neurons in birds and cold-blooded animals.

In the mammalian retina, no 5-hydroxytryptamine immunoreactive neurons were detected, and, correspondingly, the concentrations of 5-hydroxytryptamine were lower. However, in the cow and pig retina, autoradiography with (^3H)-5-hydroxytryptamine revealed amacrine neurons with the ability to accumulate the substance. These cells were not seen with formaldehyde fluorescence histochemistry, which may indicate that the 5-hydroxytryptamine taken up by the cells is rapidly transformed into a non-fluorescent, still unknown, metabolite. The low concentrations of 5-hydroxytryptamine and the absence of cells with a sufficient amount of 5-hydroxytryptamine to be detected by immunohistochemistry makes this substance less likely as a transmitter in the mammalian retina.

The occurrence of retinal neurons containing peptides such as somatostatin, VIP, glucagon, enkephalin, neurotensin and substance P was investigated. Somatostatin and VIP neurons

were detected among the amacrine cells in a number of different species, including man. Generally, cell bodies were found in the innermost part of the inner nuclear layer with nerve fibres forming two or three nerve fibre layers in the inner plexiform layer. In some species, interplexiform somatostatin immunoreactive nerve fibres were noted. In the baboon retina, VIP neurons were found in the inner plexiform layer exclusively, thus constituting a special group of interstitial amacrine cells. Glucagon immunoreactivity was found in amacrine cells in retinæ from birds and cold-blooded animals, whereas enkephalin (with one exception) and neurotensin immunoreactivity was found in amacrine cells in birds only. Substance P was found in amacrine cells, in interstitial amacrine cells and in interplexiform fibres in the human retina.

The immunohistochemical findings on VIP and glucagon were supplemented by an immunochemical study wherein the concentrations of VIP and glucagon in extracts from retinæ were determined. The immunochemical findings were, on the whole, in good agreement with immunohistochemical observations. Furthermore, the VIP and glucagon immunoreactive material was characterized by chromatography. It was found that VIP extracted from bovine retinæ could not be distinguished from porcine (^{125}I)-labelled VIP by gel chromatography. Glucagon immunoreactive material extracted from chicken and frog retinæ had the same elution position as (^{125}I)-labelled and pure porcine pancreatic-type glucagon in gel chromatography and with HPLC.

To investigate the possible co-occurrence of an established transmitter and a peptide, a method using simultaneous autoradiography and immunohistochemistry was developed. In

different series of sections the possible co-occurrence of (^3H)-5-hydroxytryptamine and any of the peptides somatostatin, glucagon, substance P, neurotensin and enkephalin was investigated. With statistics based on the binomial distribution it was possible to state with more than 98% confidence that the true frequency of cells possibly containing both (^3H)-5-hydroxytryptamine and any of the peptides was less than 1%.

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5-Hydroxytryptamine in the Retina of some Mammals

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A set of indoleamine accumulating neurons has been described in the retina of several species. Their transmitter is unknown though 5-hydroxytryptamine has been suggested. We have studied the 5-hydroxytryptamine concentration in the retina of cows, pigs, guinea-pigs and also of rabbits in which the blood had been washed out. It was found to be 26.2 ng/g (S.E.M. = 7.17) in cow retina, 5.9 (S.E.M. = 0.83) in pig retina and 6.03 ng/g (S.E.M. = 2.61) in guinea-pig retina. Concentrations in this range could well be due to blood platelet 5-hydroxytryptamine and this was corroborated by the about six-fold fall from the normal concentration to 3.8 ng/g (S.E.M. = 0.9) which occurred when rabbit retinas were perfused to remove the blood. The possible presence of indoleamine accumulating neurons in some mammals (cow and pig) was also investigated by means of fluorescence microscopy. No indoleamine accumulating neurons could be found with this technique. Nevertheless, autoradiography has shown that there are indoleamine accumulating neurons in the cow and pig retina, and we suggest that these neurons have the ability to modify the indoleamines they accumulate into substances related to 5-hydroxytryptamine but unable to form fluorophores with formaldehyde in tissues.

Key words: indoleamine accumulating neurons; 5-hydroxytryptamine; retina; fluorescence microscopy; high performance liquid chromatography (HPLC); cow; pig; guinea-pig; rabbit.

1. Introduction

Recently a set of indoleaminergic neurons in the retina of certain species (rabbit, cat, goldfish, chicken and pigeon) has been described (Ehinger and Florén, 1976; Florén, 1979). These neurons are characterized by their ability to accumulate indoleamines and consequently they could be visualized by the histofluorescence technique of Falck and Hillarp. The processes of these neurons are situated in the inner plexiform layer with their perikarya localized to the innermost part of the inner nuclear layer, i.e. their localization agrees with that of amacrine cells. Their function is still unknown and so is their transmitter.

5-Hydroxytryptamine (5-HT), an indoleamine and a well-known transmitter of the central nervous system, has been mentioned as a possible transmitter candidate of the indoleamine accumulating neurons (Ehinger and Florén, 1978; Thomas and Redburn, 1979; Osborne, 1980). It is present in the retina of rabbit and chicken as well as in the bovine retina in small amounts (Florén and Hansson, 1980; Osborne, 1980). However, 5-hydroxytryptamine is a substance that occurs also outside the CNS, for instance in the thrombocytes of the blood, and it is possible that at least a part of the 5-hydroxytryptamine found in the retina derives from the thrombocytes. The aim of this study was therefore to repeat the 5-hydroxytryptamine determinations in rabbit retina after blood removal by perfusion. Moreover, the 5-hydroxytryptamine concentrations in retina of some other mammals (cow, pig and guinea-pig) have been determined and attempts have been made to demonstrate indoleamine accumulating neurons by fluorescence microscopy of the retina of these animals.

The results have in part been presented in preliminary form in a review (Ehinger and Florén, 1980).

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2. Materials and Methods

Catheterization and perfusion

Ordinary pigmented rabbits of either sex and weighing about 2 kg were anesthetized with an intraperitoneal injection of Mebumal® whereafter the thorax was opened and the aorta was catheterized while the animal was kept on an ice bed. The upper half of the body was then pressure perfused with ice-cold Ringer-glucose (containing per litre: 73.5 meq Na⁺, 2.3 meq Ca²⁺, 2 meq K⁺, 77.8 meq Cl⁻ and 153 mmol glucose, giving 285 mosmo/1000 g H₂O) until the fluid leaving the right atrium was clear. Ordinarily about 1 l of Ringer-glucose was needed. Ordinary pigmented guinea-pigs were killed with ether. Retinas from cow and pig were obtained from a nearby slaughter-house less than 5 min after the slaughter. Retinas were also taken from juvenile pigs (10 kg) killed in the laboratory. All the retinas were kept in liquid nitrogen until the 5-hydroxytryptamine analyses were performed, always within 24 hr.

5-Hydroxytryptamine analysis

The analysis was performed by high-performance liquid chromatography (HPLC) according to the method described by Hansson and Rosengren (1978). The tissue samples were homogenized in 2 ml 0.4 M-HClO₄ together with 100 ng of N-methyl-5-hydroxytryptamine which served as internal standard. After centrifugation and adjustment of pH to 6.0 the samples were purified and concentrated by passing them through a weakly acidic cation exchange resin (Amberlite IRP-64). 5-Hydroxytryptamine as well as other amines and the internal standard were then eluted with 2 × 0.5 ml HCl. One hundred microlitres of the eluted volume was chromatographed on microparticulate reversed phase packing material with chemically bonded octadecyl groups on 5 µm silica using a high-pressure pump and an electrochemical detector. The limit of detection for 5-hydroxytryptamine in this system is about 25 pg which with the tissue amounts usually used (about 80–150 mg) gives a lower level of detectability of about 0.15–0.40 ng/g. The two retinas from each rabbit were pooled and five guinea-pig retinas were pooled for each estimate. Retinas from cow and pig could be analysed individually.

Histochemistry

The retinas were obtained in a nearby slaughter-house and transported to the laboratory in ice-cold balanced salt solution (see Ames, Parks and Nesbett, 1980). Twenty or fifty micrograms 5,6-dihydroxytryptamine was also injected into the vitreous of the juvenile pigs and the retinas were processed 4 hr later. The drug was dissolved in 0.9 % NaCl with ascorbic acid (1 mg/ml) added as an antioxidant. All retinas were processed for the demonstration of catecholamines and indoleamines in the fluorescence microscope according to the method of Falck and Hillarp (Falck and Owman, 1965; Björklund, Falck and Owman, 1972). The tissue samples were freeze-dried, exposed to formaldehyde for 1 hr at 80°C (whereby the fluorophores were formed), embedded in vacuo in paraffin wax and sectioned. In the fluorescence microscope dopaminergic neurons will show a blue or greenish fluorescence (depending on the barrier filter) while indoleamine neurons show yellow fluorescence.

3. Results

5-Hydroxytryptamine estimates

A total of 21 rabbits were perfused (Table I). In this group the 5-hydroxytryptamine content was below the limit of detection in seven samples. In two samples the 5-hydroxytryptamine content was very high, 77.5 and 78.5 ng/g, respectively, whereas the 5-hydroxytryptamine content in the remaining samples varied between 1.3 and 15.6 ng/g. The two exceptionally high figures are not included in Table I. We had problems with blood clotting and incomplete perfusions in these two experiments and presumably thrombocyte plugs were formed during perfusion. If they are nevertheless included, the arithmetic mean becomes 10.9 (s.e.m. = 4.9) and the median

TABLE I

5-Hydroxytryptamine concentrations (ng/g wet weight) in the retina

	High-pressure liquid chromatography			
	Average $\pm$ S.E.M.	Median	Number of assays below level of detectability	Number of assays
Normal rabbit*	24 $\pm$ 5†‡	18	0	17
Perfused (bloodless) rabbit§†	3.8 $\pm$ 0.9†	2.3	7	19§
Cow	26.2 $\pm$ 7.17	17.1	0	16
Pig (adult)	5.9 $\pm$ 0.83‡	4.9	1	16
Guinea-pig	6.03 $\pm$ 2.61‡	3.8	0	6

* Figures from Florén and Hansson (1978).

† and ‡ significantly different by Wilcoxon rank test ($P < 0.01$).

§ Two estimates not included. See the text.

2.4 for the perfused retinas. The Wilcoxon rank test for difference between normal and perfused rabbits remains significant ($P < 0.01$). The values below detection level occurred scattered irregularly throughout the study.

Six samples, each with five retinas from guinea-pigs were analysed. The content varied between 1.6 and 18.7 ng/g, with a mean value of 6.03 ± 2.6 ng (Table I).

Retinas from cow and pig contained enough tissue for separate analysis of each retina. In the bovine retina the 5-hydroxytryptamine content appeared to be in the same range as in non-perfused rabbits and somewhat higher than in the other species examined (Table I).

Histochemistry

Normal retinas from pig and cow treated with the histofluorescence method of Falck and Hillarp contain a number of neurons with greenish fluorescence. Their terminals form a dense network confined to a narrow sublayer at the border between the inner nuclear and inner plexiform layers (Fig. 1). The sparsely occurring cell bodies from which these terminals emerge are situated in the innermost third of the inner nuclear layer. When situated in the innermost cell row, the cell bodies are usually rounded while further outwards they are slightly smaller and more oval or drop-shaped with their long axis perpendicular to the retinal surface. In a few cases a fluorescent terminal could be traced outwards through the inner nuclear layer but never further than halfway through. A few fibres were also infrequently detected in the middle and inner thirds of the inner plexiform layers. Otherwise, the retina did not have any fluorescent terminals or cell bodies. Incubating the retina in alpha-methylnoradrenaline (1 or 10 μ g/ml) increased the fluorescence intensity of the neurons described above (Figs 2–4) but did not cause any additional ones to appear. The morphology of these cells correspond closely to the dopaminergic amacrine cells previously found in a number of other animals (see Ehinger, 1978) and will therefore be referred to as such cells. Fluorescence was at times seen in blood vessels, in the nerve fibre layer and in the most vitreal parts of the glial cells with the highest doses of alpha-methylnoradrenaline (Fig. 4). There were no neurons or other structures in the

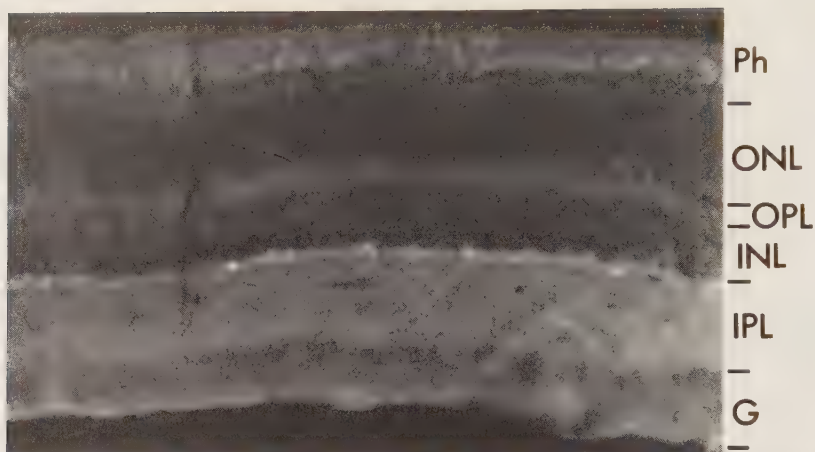


FIG. 1. Normal retina from pig. There is a single narrow layer of fluorescent nerve fibres at the border between the inner nuclear layer and the inner plexiform layer. Cell bodies are rare and none is shown. Ph, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Fluorescence micrograph, $\times 190$.



FIG. 2. Cow retina incubated in α -methylnoradrenaline, $1 \mu\text{g/ml}$. The fluorescence in the narrow layer of nerve fibres has been intensified but no additional fluorescent neurons occur. Above, fluorescence micrograph; below, phase contrast micrograph of the same area. Designation of layers as in Fig. 1, $\times 255$.

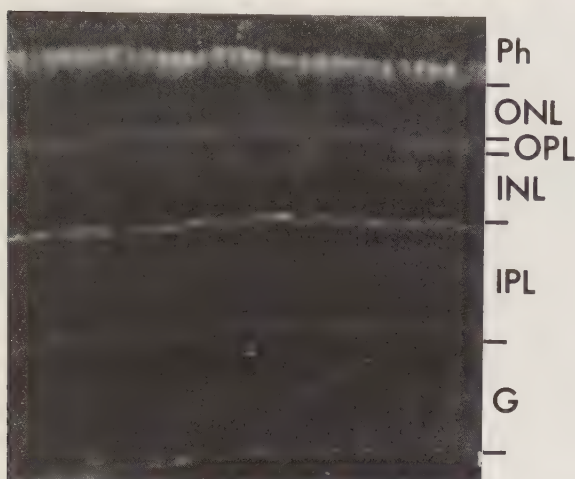


FIG. 3. Pig retina incubated in alpha-methylnoradrenaline, $1 \mu\text{g/ml}$. As in the cow retina the normally occurring layer of fluorescent nerve fibres at the border between the inner nuclear layer and inner plexiform layer has become more intense than in the normal retina but no additional neurons occur. Designation of layers as in Fig. 1. Fluorescence micrograph, $\times 205$.

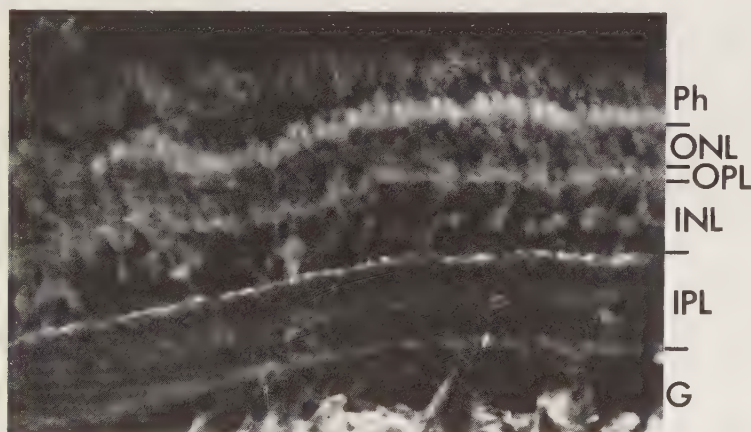


FIG. 4. Pig retina after incubation in alpha-methylnoradrenaline, $10 \mu\text{g/ml}$. Fluorescence is seen in a nerve fibre layer at the border between the inner nuclear layer and the inner plexiform layer. Non-specific fluorescence occurs in capillary walls and glia, in the ganglion cell and nerve fibre layers but no additional neurons occur. Designations of layers as in Fig. 1. Fluorescence micrograph, $\times 210$.

normal retina which displayed yellowish formaldehyde induced fluorescence that could suggest the presence of an indoleamine.

After incubation in 5,6-dihydroxytryptamine or the intravitreal injection of the drug (Fig. 5) the dopaminergic cells described above attained the yellowish fluorescence typical for structures containing an indoleamine. The number of neurons demonstrable in the fluorescence microscope did not increase with the lower doses used. Neither did with the higher doses any fluorescence appear in neurons similar to the indoleamine accumulating ones in rabbits. However, pericytes around the small vessels then became fluorescent and a diffuse, widespread similar fluorescence appeared in the

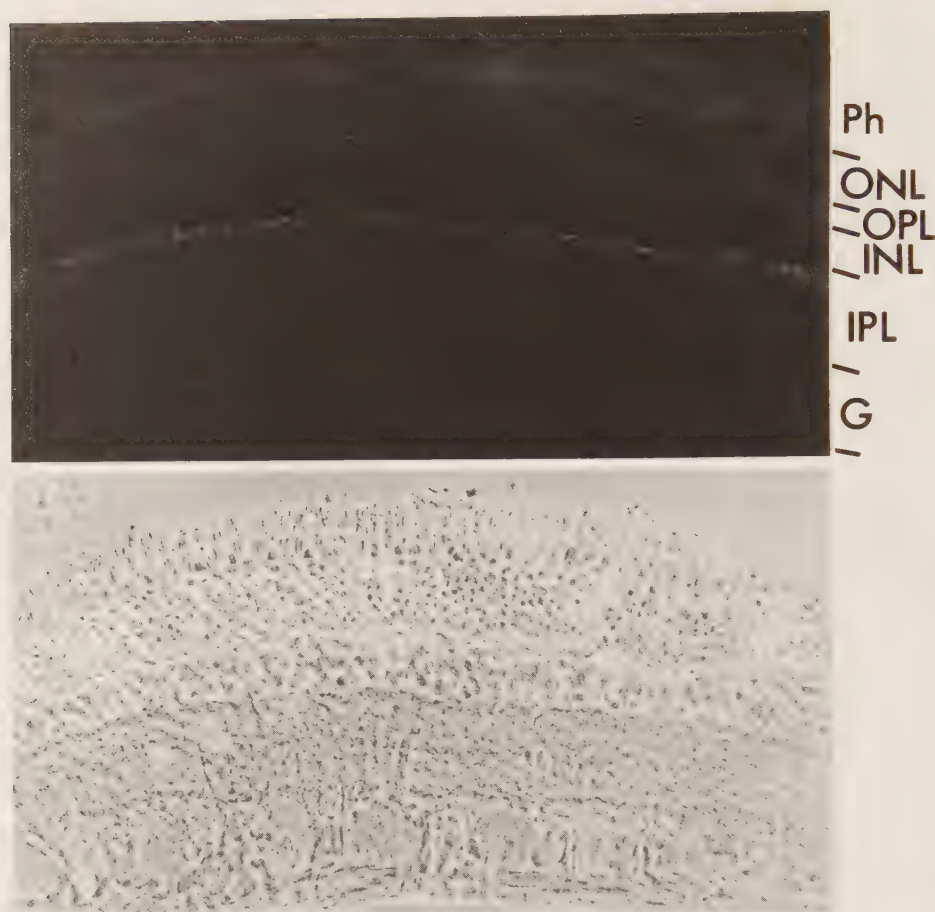


FIG. 5. Cow retina incubated in 5,6 dihydroxytryptamine, $1 \mu\text{g/ml}$. The normally occurring greenish fluorescence in a narrow layer at the border between the inner nuclear layer and the inner plexiform layer has turned yellowish but no additional neurons occur. Above, fluorescence micrograph; below, phase contrast micrograph of the same area. Designation of layers as in Fig. 1, $\times 205$.

innermost layers of the retina without association to any particular structure. Further, cell bodies with a yellowish fluorescence could not be seen in the middle of the inner nuclear layer, sending fine branches towards both the inner plexiform and outer plexiform layers, apparently terminating close to the external horizontal cells and among the dopaminergic terminals in the inner plexiform layer. These cells will be referred to as bipolar-like. Weak yellowish fluorescence was then also seen in most other neurons in the retina. Fluorescence was then also present in the parts of the glial cells closest to the vitreous (Fig. 6).

4. Discussion

The cells displaying greenish fluorescence in normal cow and pig retina or after loading with a catecholamine have a location and shape which is very similar to the dopaminergic amacrine cells which are well known in a number of other species (see



FIG. 6. Cow retina incubated in 5,6 dihydroxytryptamine $30 \mu\text{g/ml}$. Apart from fluorescence in the nerve fibre layer at the border between the inner nuclear layer and the inner plexiform layer non-specific fluorescence now occurs in bipolar-like cells in the inner nuclear layer and in glial cells in the innermost part of the retina. However, no additional neuronal elements occur. Designation of layers as in Fig. 1. Fluorescence micrograph, $\times 205$.

Ehinger, 1978). The terminal processes of these cells are stratified and form one to three sublayers in the inner plexiform layer in different species. Humans, Old World monkeys, cats and rats have only one sublayer and cows as well as pigs belong to this group. Rabbits have three sublayers and guinea-pigs have two (Ehinger, 1978). The significance of these species differences is not clear but it may be noted that synapses mediating on and off responses have been shown to be located in different sublayers in the retina of cats (Nelson, Famiglietti and Kolb, 1978) and mudpuppies (Miller and Dacheux, 1976).

A special variety of neurons, the dopaminergic interplexiform cells, have been found in certain New World monkeys and in teleost fish. We were unable to detect such neurons in cows and pigs, even after loading the retinas with a catecholamine. It can be concluded that, as with a number of other mammals, (see Ehinger, 1978) if interplexiform cells occur in pigs or cows they most likely do not use dopamine or any related substance as their transmitter.

Exposing cow and pig retinas to moderate doses of indoleamines did not result in any neuronal uptake except in the dopaminergic neurons which attained the yellowish fluorescence typical of indoleamines. With higher doses (30 or $50 \mu\text{g/ml}$) fluorescence began to appear in glia, in capillary walls and in neurons which resemble bipolars. The accumulation in capillary walls has been seen both with 5,6-dihydroxytryptamine and with other monoamines in ordinary laboratory animals (Bertler, Falck and Rosengren, 1963; Ehinger and Falck, 1969; Ehinger and Florén, 1979) and has been regarded as an effect of the blood-brain barrier system, excluding the amines from the brain. The bipolar-like neurons appearing with the high doses of 5,6-dihydroxytryptamine are reminiscent of the ones seen under similar conditions in chicken and pigeon (Florén, 1979). The comparatively high doses necessary to bring out these cells make the significance of the accumulation somewhat doubtful.

The attempts in cows and pigs to identify indoleamine accumulating neurons comparable with the ones in rabbits have thus failed to show any. This is also the case

in rats (Florén, 1978) and cynomolgus monkeys (Ehinger and Florén, 1979). In rabbits, on the other hand, the procedures used here invariably demonstrate the indoleamine accumulating neurons (Ehinger and Florén, 1976) and control runs have repeatedly been done with rabbit retinas to check the procedure.

In a previous study the 5-hydroxytryptamine concentration was found to be 24 ± 5 ng/g wet weight in the rabbit retina, and it was found that this could well represent blood (platelet) 5-hydroxytryptamine (Florén and Hansson, 1980). This is corroborated by the present study where less than the detection limit (about 0.5 ng/g) was found in 7 out of 19 retinas from which the blood had been washed out and a fall to an average of 3.8 ± 0.9 ng/g in 5-hydroxytryptamine content upon washing. The difference is significant ($P < 0.01$, Wilcoxon rank correlation test).

The content of 5-hydroxytryptamine in the guinea-pig retina was found to be significantly lower than in the normal rabbit retina. The blood content of 5-hydroxytryptamine is about 10 times lower in guinea-pig than in rabbits and this may, in part, explain the difference. Other factors, such as degree of vascularization of the retina, may also enter because the blood 5-hydroxytryptamine is similar in cow and pig (Drumond, 1976); yet there is a difference in retinal 5-hydroxytryptamine content.

The present figures agree with those of Häggendal and Malmfors (1965), who were unable to detect 5-hydroxytryptamine, with Osborn's (1980) and with our previous observations (Florén and Hansson, 1980). However, they are significantly lower than those of Thomas and Redburn (1979) who reported 100 ± 10 ng/g in cows, or Suzuki, Noguchi, Miyake and Yagi (1977), which found 176 ng/g in chicken retina. The reason for this is not clear but it may be technical. We have no reason to assume degradation in our HPLC test system because test analyses of rabbit midbrain 5-hydroxytryptamine yielded values in the expected range, about a tenfold higher than in the retina (Florén and Hansson, 1980). We find it more likely that the previous fluorometric assays include not only 5-hydroxytryptamine but also the unknown transmitter of the indoleamine accumulating neurons, which we presume is a substance related to 5-hydroxytryptamine (see the following discussion). It is further worth noticing that the lower limit of detection with HPLC with an electrochemical detector is at about 25 pg (Hansson and Rosengren, 1978; Ponzio and Jonsson, 1979; Koch and Kissinger, 1980) whereas fluorometric methods usually have a limit of detection at about 40 ng (e.g. Thomas and Redburn, 1979). Blank errors are thus much more likely to affect the fluorimetry than the HPLC when measuring 100–500 mg tissue with a concentration ranging perhaps from 10 to 100 ng/g wet weight (i.e. measuring total amounts of 1–50 ng).

The experiments reported here do not favour the hypothesis that 5-hydroxytryptamine is a retinal neurotransmitter: the concentration is too low, much of it can be washed out with the blood (where the thrombocytes are known to contain 5-hydroxytryptamine), and fluorescence microscopy fails to show any indoleamine accumulating neurons in a number of species including humans, the cynomolgus monkey (Ehinger and Florén, 1979), cows, pigs (this study), guinea-pigs and rats (Florén, 1978). However, [^3H]-5-hydroxytryptamine yields radioactivity in a distinct set of amacrine neurons in retina from cow (Osborne 1980; Ehinger, Florén and Tornqvist, 1981) and pig but not in retinas from rats, cynomolgus monkeys, baboons and humans (Ehinger, Florén and Tornqvist, 1981), and these neurons are thus indoleamine accumulating but not demonstrable with fluorescence microscopy. If 5,6-dihydroxytryptamine is accumulated in these neurons it is apparently rapidly transformed into a form not able to form fluorophores with formaldehyde in tissues.

We hypothesize from these discrepancies and the differences in concentration obtained with different analytical techniques that there is a set of amacrine neurons in the retina of most species that operate with a 5-hydroxytryptamine-like transmitter and that in certain species these neurons have uptake mechanisms that make it possible to demonstrate them with indoles such as 5-hydroxytryptamine or 5,6-dihydroxytryptamine.

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Autoradiography of (³H)-5-Hydroxytryptamine Uptake in the Retina of Some Mammals

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Abstract. The uptake of indoleamines into the retina of rats, rabbits, cows, pigs, baboons, Cynomolgus monkeys, and man was studied by fluorescence microscopy and autoradiography. Indoleamines were either injected intravitreally or the retinas were incubated with them. Fluorescence microscopy failed to show any indoleamine accumulating neurons in all species investigated except rabbit, confirming previous observations. However, autoradiography showed uptake in a distinct class of neurons in cows and pigs. These neurons had their cell bodies among the amacrine cells and most of their processes branched in the middle of the inner plexiform layer. This is in contradistinction to the dopaminergic neurons, which in cows and pigs have all their processes in the outermost sublamina of the inner plexiform layer. The fluorescence microscopy is quite sensitive to small variations in the indoleamine molecule. The discrepancy between the results with fluorescence microscopy and autoradiography therefore suggests that there is an active uptake mechanism for indoleamines in cows and pigs but that the substances are rapidly transformed to compounds not possible to detect in the fluorescence microscope. No specific indoleamine accumulating mechanism was detected in the retina of rats, baboons, cynomolgus monkeys, or man.

Introduction

Indoleamine accumulating neurons have been detected in the retina of several species (Ehinger and Florén 1976, 1980), but their transmitter is not known, even if it seems reasonable to assume it is a substance similar to 5-hydroxytryptamine or possibly identical with it. Uptake systems for indoleamines were originally found in the rabbit retina (Ehinger and Florén 1976) and subsequently in a number of other species (lamprey: Ehinger et al. 1977; mudpuppy: Adolph et al. 1980; goldfish: Ehinger and Florén 1976; Cebus monkey: Dowling et al. 1980; squirrel monkey: Florén and Hendrickson 1980). The uptake system was biochemically characterized in the rabbit (Ehinger and Florén 1978), and a similar system was detected biochemically in the cow retina (Thomas and Redburn 1979, 1980; Osborne 1980; Osborne and Richardson 1980). However, fluorescence microscopy failed to show any neurons that would accumulate indoleamines in the retina from a number of species including cow (Ehinger and Florén 1979; Ehinger and Florén 1980; Ehinger et al. 1981), and it is therefore uncertain what the uptake reported in the cow retina represents. We have consequently reinvestigated

the uptake of indoleamines in the rat, cow, pig, and monkey retina by fluorescence microscopy, added *homo sapiens* and extended the study to include autoradiography of the uptake of (³H)-5-hydroxytryptamine in the same animals. We find that the two methods give seemingly discordant results, necessitating the conclusion that the indoleamine-accumulating neurons in the retina of at least certain species have fundamental differences from conventional 5-hydroxytryptaminergic neurons.

Materials and Methods

Retinas from the cow and pig were obtained in an abattoir less than 3 min after the slaughter of the animals. They were either immediately incubated at room temperature (20°–22° C) with the otherwise standard procedure described below or brought to the laboratory (30–45 min) immersed in the oxygenated salt solution at 0° C. Standard incubations were then performed at 37° C.

Other retinas were also incubated according to the standard procedure: four juvenile pigs (4 months), six rats, six baboons, two Cynomolgus monkeys, and six human eyes (patient ages: 48, 67, 68, 71, 73 and 80 years; all enucleated because of malignant melanoma). Intraocular injections were also given to rats and pigs (rats: 5 µg 5,6-dihydroxytryptamine or 5 or 10 µCi (³H)-5-HT; pigs: 20 or 50 µg 5,6-dihydroxytryptamine). Pieces of the retina were obtained after 4 h and processed for fluorescence microscopy or autoradiography as described below. Pieces of normal retinas were always incubated as controls and for comparison.

In the standard incubation procedure, the tissue pieces were transferred to small Erlenmeyer flasks containing 4 ml of oxygenized, balanced salt solution according to Ames (see Ames et al. 1980) at the described temperature and left for 10 min with gentle agitation. The incubation was then performed with 5,6-dihydroxytryptamine creatinine sulphate (5, 10, 25, 30, or 50 µg/ml or (³H)-5-hydroxytryptamine (1, 2, or 2.6 µCi/ml). The retinas were subsequently washed two times for 5 min in fresh salt solution without additives at 0° C and freeze-dried in a tissue dryer. They were then fixed in gaseous formaldehyde for 1 h at 80° C. Specimens for fluorescence microscopy were embedded in paraffin wax and specimens for autoradiography in plastic (Durcupan, Fluka) in vacuo. For the autoradiography, sections were covered with stripping film (Kodak AR 10) floated on distilled water and exposed for 4–12 weeks.

5,6-dihydroxytryptamine creatinine sulphate was obtained from Regis Chemical Co., Morton Grove, Illinois; 5-(1,2-(³H)-(N))-hydroxytryptamine, specific activity 500 mCi/mmol, from New England Nuclear Co, Dreieichenhain, Germany.

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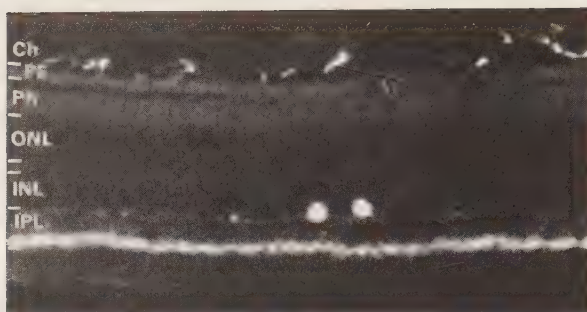


Fig. 1. Rabbit retina 4 h after the intravitreal injection of 50 μ g 5,6-dihydroxytryptamine. There is intense fluorescence in a broad sublayer of tightly intertwined, fine varicose terminals in the innermost part of the inner plexiform layer (IPL) and a less dense network of fibres in the rest of the IPL. There are two fluorescent cell bodies located in the innermost part of the inner nuclear layer (INL). There are strongly fluorescent nerve terminals in the choroid (Ch). Fluorescence micrograph, Falck-Hillarp procedure. $\times 280$

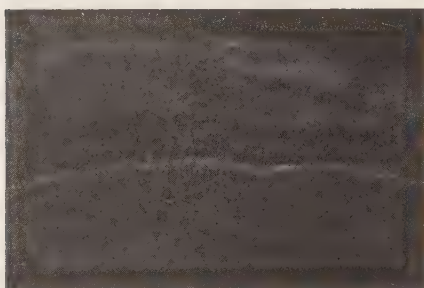


Fig. 2. Cow retina, incubated 15 min at 37°C in 5,6-dihydroxytryptamine, 1 μ g/ml. There is a single band of yellowish, fluorescent fibres at the border between the inner nuclear (INL) and inner plexiform layers (IPL). Above, fluorescence micrograph, Falck-Hillarp procedure; below, phase contrast micrograph of the same region. $\times 200$

Results

Fluorescence Microscopy

Rabbit. The distribution of neurons accumulating 5,6-dihydroxytryptamine is shown in Fig. 1. This has been published previously (Ehinger and Florén 1976), and the picture is included here for comparison only. Equal results were obtained with 6-hydroxytryptamine or 5,7-dihydroxytryptamine (both given intravitreally, 50 μ g). 5-methoxytryptamine will also give this picture, although with considerably less fluorescence intensity, as will 5-hydroxytryptophan (50 μ g intravitreally) in animals pretreated with the monoamine oxidase inhibitor, Pargyline (50 mg/kg IP).

Rat, Cow, Pig, Baboon, Cynomolgus Monkey, Man (Figs. 2–4). In all these cases, the previously described dopaminergic neurons (Ehinger 1966a and b, 1976; Ehinger et al. 1981), showed yellow fluorescence, indicating they had accumulated 5,6-dihydroxytryptamine. In all species there was an increased general glial fluorescence, particularly in the innermost parts of the pig retina. Capillaries often showed yellowish fluorescence, as has been noted previously (Ehinger and Florén 1979). No additional neurons appeared in any of these species with low-to-moderate concentrations of 5,6-dihydroxytryptamine (less than 30 μ g/ml). In particular, we were not able to see nerve terminals in cow and pig retinas in the sublayers of the inner plexiform layer where the autoradiography (see below) of parallel incubations of pieces of the same retinas in (3 H)-5-hydroxytryptamine showed accumulation of radioactivity. With the highest concentrations, certain bipolarlike cells became fluorescent (not illustrated), as has been previously described in some species (Florén 1979; Ehinger and Florén 1980; Ehinger et al. 1981).

Autoradiography

Rabbits. After intraocular injections of (3 H)-5-hydroxytryptamine, radioactivity appeared (Fig. 5) in a well-developed band in the inner part of the inner plexiform layer (sublamina 5) and at times also in two not very well demarcated sublayers, one near the border to the inner nuclear layer (in sublamina 1) and one near the middle of the inner plexiform layer (sublamina 3). The innermost layer was much more developed than the others. Radioactive cell bodies appeared in the innermost two or three cell rows of the inner nuclear layer. When the retina was thin (i.e., taken from the periphery of the eye), only the innermost cell row contained radioactive cell bodies. The perikarya situated the farthest out were more or less pearlike, but perikarya situated in the innermost cell row were more rounded.

Rat. Intraocular injection of up to 10 μ Ci of (3 H)-5-hydroxytryptamine did not result in any selective radioactivity in neurons. Instead, there was a rather diffuse radioactivity throughout the retina, less intense in the outermost parts than in the innermost ones. The pigment epithelium was always more radioactive than the choroid or the photoreceptors.

Cow. Retinas incubated in (3 H)-5-hydroxytryptamine always showed radioactivity in two sublayers in the inner plexiform layer, one close to the outer border (sublamina 1) and one in the middle of it (sublamina 3) (Fig. 6). The outermost sublayer was usually the best developed, although the difference was not

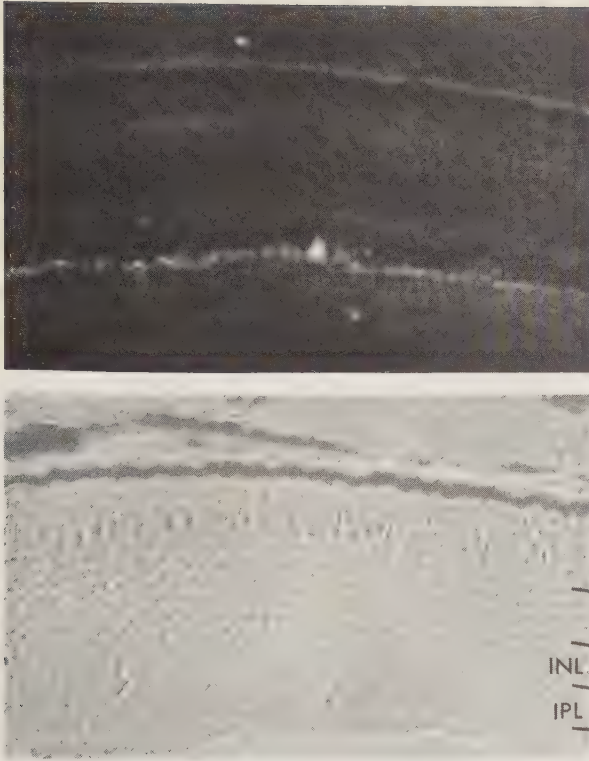


Fig. 3. Cynomolgus monkey 4 h after the intravitreal injection of 50 μ g 5,6-dihydroxytryptamine. There is a single layer of fluorescent terminals at the border between the inner nuclear (INL) and inner plexiform layers (IPL) with a few fibres reaching a short distance out among the nonfluorescent cell bodies of the INL. There is one fluorescent cell body. Above, fluorescence micrograph, Falck-Hillarp procedure; below, phase contrast micrograph of the same area. $\times 275$

always very striking. There was no appreciable radioactivity in the outer plexiform layer. No radioactivity was seen in ganglion cells, but there was some radioactivity in the glial cells. Cell bodies were frequently seen in the innermost two cell rows in the inner nuclear layer with approximately the same frequency as in pigs. They did not deviate significantly in size or shape from their neighboring cells (as seen with toluidine-blue staining or with phase-contrast optics). Radioactive cell bodies were also rarely encountered in the middle of the inner plexiform layer (Fig. 7).

Pigs. These retinas showed radioactivity in two sublayers in the inner plexiform layer, one near its outer border (sublamina 1) and one in the middle (sublamina 3). The innermost layer was usually the best developed; the outer was not always clearly distinguishable (Fig. 8). Radioactive cell bodies were frequent in the innermost two or three cell rows in the inner nuclear layer. They were often seen to send coarse radioactive processes into the inner plexiform layer, participating in forming both radioactive sublayers (Fig. 9) although the resolution in the autoradiograms is insufficient for establishing whether one cell could send processes to both sublayers. Radioactive cell bodies were also occasionally seen in the middle of the inner plexiform layer and, about equally seldom, among the ganglion cells. In the latter, they were seen to send processes down to either of the two sublayers in the inner plexiform layer.

In retinas that had detached during the incubation there was usually some radioactivity in a narrow band at the photoreceptor pedicles in the outer plexiform layer, and occasionally it was present in spots, suggestive of coarse cell processes or cell bodies (Fig. 10).

Glial cells also became radioactive to a significant extent in the pig retina, but usually mainly in the inner parts of the retina (Fig. 9). The glial radioactivity, therefore, did not disguise very much of the neuronal radioactivity. Vessel walls accumulated considerable radioactivity (Fig. 8).

Monkeys. Incubating both the baboon and the Cynomolgus monkey retinas in (3 H)-5-hydroxytryptamine resulted mainly in diffuse radioactivity throughout the retina with a slight gradient, diminishing outward. There was a single weak sublayer of radioactivity in the outermost part of the inner plexiform layer (Fig. 11) and some equally feeble cell bodies in the innermost part of the inner nuclear layer. A few radioactive cells have been observed among the ganglion cells, none in the inner plexiform layer, and none outward from the inner nuclear layer.

Homo sapiens. These retinas showed considerable degenerative senile changes with much vacuolation in the middle layers. To some degree this is likely to be incubation artifacts, but these changes were also seen to a considerable extent in retinas fixed without incubating them first. As in the monkey, there was



Fig. 4. Human retina, incubated for 15 min at 37° C in 5,6-dihydroxytryptamine, 50 µg/ml. There is only a single layer of fluorescent terminals at the border between the inner nuclear (INL) and inner plexiform (IPL) layers. Above, fluorescence micrograph, Falck-Hillarp procedure; below, phase contrast micrograph of the same area. ×280



Fig. 5. Rabbit retina 4 h after the intravitreal injection of 20 µCi (³H)-5-HT. The distribution of the radioactivity agrees very well with the fluorescence picture (compare with Fig. 1). Phase contrast micrographs. Left, focus on the section; right, focus on the silver grains ×250

considerable diffuse radioactivity and only weak radioactivity in a single sublayer in sublamina 1 in the inner plexiform layer (Fig. 12), together with a few radioactive cell bodies, most of which were among the amacrine cells and a few among the ganglion cells.

Discussion

The results show that indoleamine-accumulating neurons cannot be demonstrated with the fluorescence microscope in the rat, *Cynomolgus* monkey, baboon, pig, cow, or in man and they

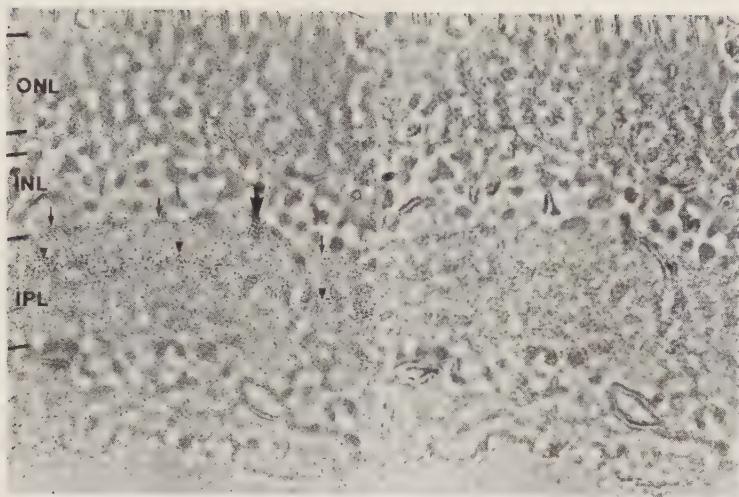


Fig. 6. Cow retina incubated in (^3H)-5-HT, 5 $\mu\text{Ci/ml}$ for 15 min at 37°C. There is radioactivity in two bands in the inner plexiform layer (IPL), one near the border to the inner nuclear layer (arrows) and one near the middle of the inner plexiform layer (arrowheads). There is also a radioactive cell body (open arrow). INL, inner nuclear layer; IPL, inner plexiform layer. Phase contrast micrographs: left, focus on the silver grains; right, focus on the section. $\times 435$

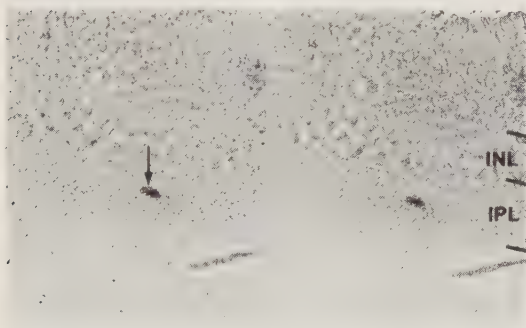


Fig. 7. Cow retina incubated in (^3H)-5-HT, 5 $\mu\text{Ci/ml}$ for 15 min at 37°C. A cell body (arrow) in the middle of the inner plexiform layer (IPL) is strongly radioactive. INL, inner nuclear layer. Phase contrast micrographs: left, focus on the silver grains; right, focus on the section. $\times 300$

thus also confirm our previous contentions (Ehinger and Florén 1979; Ehinger et al. 1981). When the retina from these species is exposed to an indoleamine such as 5,6-dihydroxytryptamine, all that happens is that the dopaminergic neurons accumulate the drug and show the yellowish fluorescence typical of indoleamines in these species. It is of some importance to note that the dopaminergic neurons become yellowish because it shows that we are not unable to demonstrate neuronal indoleamine fluorescence in these species. However, the autoradiography unequivocally shows that there are (^3H)-5-hydroxytryptamine-accumulating neurons in at least cows and pigs, which fits with the observation by Thomas and Redburn (1979, 1980), Osborne (1980), and by Osborne and Richardson (1980) of a neuronal uptake of 5-hydroxytryptamine in cows.

The difference between the results with fluorescence microscopy and autoradiography is noteworthy. Three explanations

are possible: technical failures, inability of hydroxytryptamines to form fluorophores in the tissues used, and conversion of the hydroxytryptamines to substances that will not form fluorophores with formaldehyde. The first alternative, technical failure, can be ruled out because control retinas run in parallel showed the expected pictures (usually normal animals were used as controls, and the normal dopaminergic neurons were seen in these). Moreover, the yellowish fluorescence seen in the dopaminergic neurons asserts that it is not impossible to obtain fluorophores from indoleamines in retinal neurons in the species investigated. The second alternative is also unlikely because fluorophores were formed in glia and capillaries when concentrations were high enough to force the hydroxytryptamine into them. Furthermore, the beta-carbolines formed as fluorophores from hydroxytryptamines have never been found to have tautomeric forms with unfavorable fluorescence characteristics like the quinolines

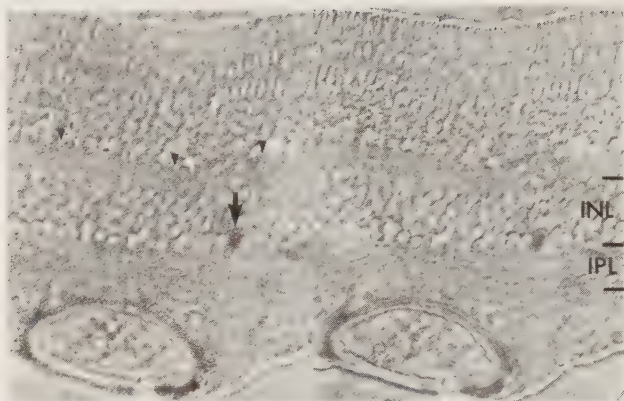


Fig. 8. Pig retina incubated in (^3H)-5-HT, 2.3 $\mu\text{Ci}/\text{ml}$ for 15 min at 37° C. The retina had detached during the incubation. There is radioactivity in the middle of the inner plexiform layer (IPL) and also diffusely throughout the IPL. A cell body among the amacrine cells is strongly radioactive (arrow). There is also some radioactivity near the photoreceptor synapses (small arrowheads). A large blood vessel is surrounded by strong radioactivity. Phase contrast micrographs: *left*, focus on the silver grains; *right*, focus on the section. $\times 290$

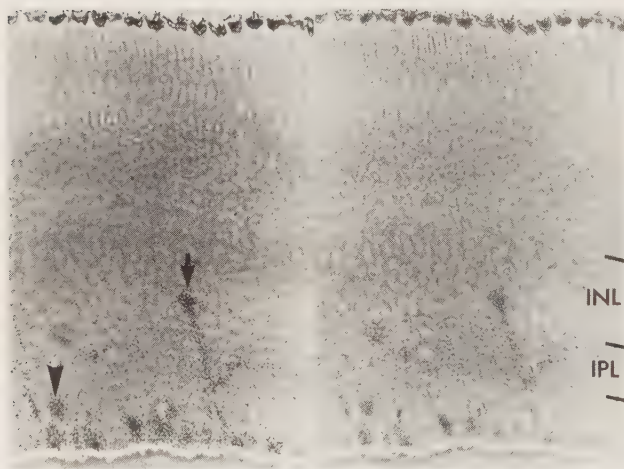


Fig. 9. Pig retina incubated in (^3H)-5-HT, 2.3 $\mu\text{Ci}/\text{ml}$ for 15 min at 37° C. This retina did not detach during incubation. There is a strongly radioactive cell body (arrow) among the amacrine cells, sending a thick process deep into the inner plexiform layer (IPL). There is also a radioactive ganglion cell (arrowhead) and radioactivity in the innermost parts of the glial cells (near the bottom edge of the retina). Phase contrast micrographs: *left*, focus on the silver grains; *right*, focus on the section. $\times 290$

formed from certain catecholamines (Björklund et al. 1972). It therefore seems most likely that the hydroxytryptamines are taken up, but subsequently metabolized. The fluorescence technique is very sensitive to certain such molecular modifications. N-methylation of the side chain completely prevents fluorophore formation (examples: melatonin, bufotenin) as does substitutes in position 1 on the ring structure. Hydroxylation in the 5-position is also a prerequisite for good fluorophore formation, whereas at least hydroxylation or methoxylation in the 4, 6, and 7 positions are of minor consequence (see Björklund et al. 1972). Hydroxylation or carboxylation of the alpha or beta carbons of the side chain likewise do not affect fluorophore formation. Melatonin can be formed by the retina and is substituted on the side chain nitrogen and methoxylated in the 5-position on the ring so that it is not demonstrable by fluorescence microscopy. The rate of formation is reported to be low, however, (Smith and Baker 1974; Wainwright 1979), and it therefore re-

mains uncertain whether conversion of indoleamines to melatonin are responsible for the changes postulated here. The melatonin concentration in the retina is also low (Pang et al. 1977), making this substance less likely as neurotransmitter.

It was previously suggested that the transmitter of the indoleamine-accumulating neurons was not 5-hydroxytryptamine, and the absence in some species of an uptake mechanism was used as one of the arguments (Ehinger and Florén 1979). The present work shows that the cow or pig retina cannot be included in these examples. However, it confirms that no indoleamine-uptake mechanism in the rat, Old World monkey, or in man is demonstrable with either fluorescence microscopy or autoradiography, and this reinforces the previous contention (Ehinger and Florén 1979) that the retinal indoleamine neurons differ from the conventional 5-hydroxytryptaminergic neurons of the brain in the way hydroxyindoleamines are handled, making 5-hydroxytryptamine an unlikely transmitter.

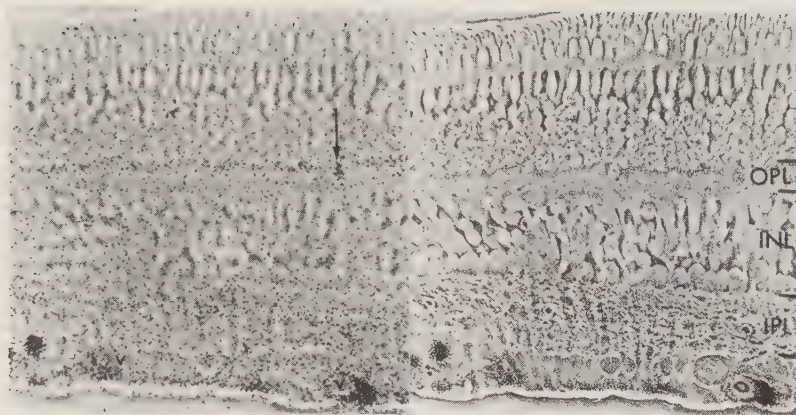


Fig. 10. Pig retina incubated in (^3H)-5-HT, 2.3 $\mu\text{Ci/ml}$ for 15 min at 37° C. The retina had detached during the incubation. There is radioactivity throughout the inner plexiform layer (IPL) with a tendency to form a sublayer of radioactivity in its middle. Pericytes around some blood vessels (v) are strongly radioactive. In the outer plexiform layer (OPL) there is radioactivity near the photoreceptor synapses and also a small accumulation of more intense radioactivity (arrow). Phase contrast micrographs: *left*, focus on the silver grains; *right*, focus on the section. $\times 435$

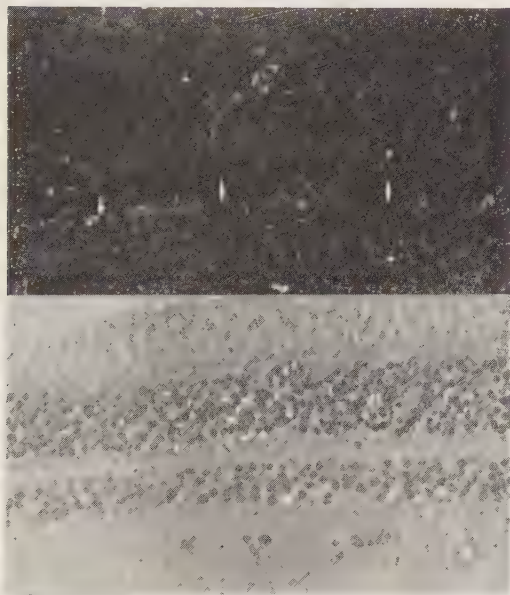


Fig. 11. Baboon retina incubated in (^3H)-5-HT, 1.3 $\mu\text{Ci/ml}$ for 15 min at 37° C. Most of the radioactivity is diffusely distributed, but there is a faint layer of increased radioactivity at the border between the inner nuclear and inner plexiform layers (arrows). *Above*, dark field micrograph; *below*, bright field micrograph of the same field, stained with toluidine blue. $\times 280$

The distribution and number of indoleamine accumulating neurons of cow and pig are similar to that seen in rabbits or chicken, for example, with the cell bodies among the amacrine cells and a band of radioactivity in the middle of the inner plexiform layer, which most likely denotes processes of the neurons. The radioactivity at the border between the inner nuclear

and the inner plexiform layer may represent uptake in indoleamine-accumulating neurons, in dopaminergic neurons, or both. In the rabbit, the last alternative is true, and this may then be the case also in the cow and pig.

The observation of radioactivity near the photoreceptor terminals is intriguing because this is the location one would expect

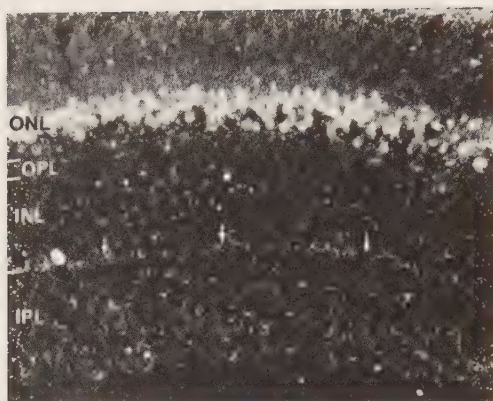


Fig. 12. Human retina (patient age: 67 years) incubated in (^3H)-5-HT, 5 $\mu\text{Ci/ml}$ for 15 min at 37° C. Like in the monkey (Fig. 11), most of the radioactivity is diffusely distributed, but there is a faint band of radioactivity at the border between the inner nuclear (INL) and inner plexiform (IPL) layers (arrows). The brightness of the photoreceptor nuclei in the outer nuclear layer (ONL) is an optical artifact. Dark field micrograph, $\times 280$

were it in interplexiform cells (see Dowling and Ehinger 1978). However, such cells have not been described yet in the cow or pig nor have any indoleamine accumulating interplexiform cells been suggested in any other species. Therefore the question must be left open as to whether there are indoleamine accumulating interplexiform cells in the pig. Florén and Hendrickson have recently (1980) demonstrated indoleamine-accumulating neurons in the horizontal cells of squirrel monkeys. However, these cells accumulated 5-hydroxytryptamine much more readily than the cells seen in pigs and had a distinctly different morphology with well demonstrable perikarya; they are, therefore, not likely to be identical with the structures seen at the photoreceptors in this study.

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IMMUNOHISTOCHEMICAL AND QUANTITATIVE ANALYSIS OF 5-HYDROXY-
TRYPTAMINE IN THE RETINA OF SOME VERTEBRATES

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KEYWORDS 5-hydroxytryptamine, retina, amacrine cells, immuno-histochemistry, high performance liquid chromatography (HPLC), vertebrates.

SUMMARY 5-hydroxytryptamine immunoreactive neurons were found in retinae from chicken, pigeon, frog and goldfish. They were localized among the amacrine cells with a distribution of cell bodies and nerve fibres that varied with the species. In chicken and pigeon, bipolar-like cell bodies were also found in the middle of the inner nuclear layer, sending processes inwards to the inner plexiform layer and outwards to the horizontal cells. The signalling direction of these cells is doubtful. No 5-hydroxytryptamine immunoreactivity was found in retinae from cow, pig, cat, rabbit, guinea-pig, rat or mouse.

Quantitative analyses were performed with HPLC on extracts from chicken, pigeon, frog and goldfish retinae. High concentrations were found in goldfish and frog whereas less, about 100 ng/g, was observed in chicken and pigeon.

The results suggest that 5-hydroxytryptamine is the transmitter of a set of amacrine cells in cold-blooded vertebrates and perhaps also in birds. The transmitter of the indoleamine accumulating neurons of mammals remains to be further elucidated.

INTRODUCTION

A set of indoleamine accumulating neurons has been described in the retina of rabbit, cat, goldfish, chicken and pigeon (Ehinger and Florén 1976, Florén 1979). The transmitter of these neurons is still unknown. 5-hydroxytryptamine is a transmitter in the central nervous system and an indoleamine and has therefore been discussed as a possible transmitter also in the retina. However, 5-hydroxytryptamine is not a probable neurotransmitter in at least the rabbit retina as its concentration is rather low and much of it seems to be located in thrombocytes (Ehinger et al. 1981). In an effort to obtain more information on the indoleamine accumulating neurons in different species we have now analysed the presence and distribution of retinal neurons displaying immunoreactivity towards 5-hydroxytryptamine in a number of different species and also quantified the content of 5-hydroxytryptamine in retina from species where the immunohistochemical studies were positive.

MATERIALS AND METHODS

Immunohistochemistry

Retinae were obtained from chicken, pigeon (*Columba livia*), goldfish (*Carassius auratus*), frog (*Rana temporaria*), cow, pig, cat, rabbit, guinea-pig, rat and mouse, at least four animals of each species. Chicken, pigeons, frogs and goldfish were decapitated. Retinae from cow and pig were obtained in a nearby slaughter-house within two minutes of the slaughter. Cats were killed by exsanguination after pentobarbital anaesthesia and rabbits were killed by air embolization. Guinea-pigs, rats and mice were killed by an overdose of diethyl ether. Retinae were in all animals rapidly dissected out and fixed by immersion in ice-cold 4% formaldehyde (40 g paraformaldehyde in 1 l 0.1 M

phosphate buffer, pH adjusted to 7.2 with 1 N NaOH) for 24 h. They were rinsed for 24-48 h in 0.1 M phosphate buffer containing 30% sucrose. The specimens were then frozen on dry ice and sectioned (15 μ m) in a cryostat at -20°C. The sections were processed for the histochemical demonstration of 5-hydroxytryptamine by the indirect immunofluorescence technique (Coons et al. 1955).

The 5-hydroxytryptamine antiserum used was partly a gift from Dr H. W. M. Steinbusch, Amsterdam, The Netherlands. This antibody is well characterized and very sensitive to 5-hydroxytryptamine (Steinbusch et al. 1978). Commercial antiserum prepared according to the same method was obtained from Immuno Nuclear Corporation, Stillwater, Minnesota, USA. The antisera were diluted 1:500 with phosphate buffered saline containing 0.25% Triton X-100 and 0.25% human serum albumin. The site of the antigen-antibody reaction was revealed with fluorescein isothiocyanate labelled anti-rabbit Ig G (Dako Immunoglobulins, Copenhagen, Denmark) diluted 1:20 with phosphate buffered 0.9% saline containing 0.25% Triton X-100. For controls, the 5-hydroxytryptamine antiserum was preincubated for 36 h at +4°C in a 30 mM solution of either of the following substances: 5-hydroxytryptamine, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, bufotenine, 6-hydroxytryptamine, 5-methoxytryptamine, 5-methoxytryptophan, 5-hydroxytryptophan, melatonin, L-tryptophan and norharmane.

Quantitative studies

Analyses of the 5-hydroxytryptamine content in retinae from chicken, pigeon, frog and goldfish were performed according to the method described by Hansson and Rosengren (1978). The tissue samples were homogenized in 2 ml 0.4 M HClO_4 together with 100 ng of α -methyl-5-hydroxytryptamine which served as internal standard. After centrifugation and adjustment of pH to 6.0, the samples were purified and concentrated by passing them through a weakly acidic cation exchange resin (Amberlite IRP-64). 5-hydroxytryptamine as well as other amines and the internal standard were then eluted with 2 ml 1.2 M HCl . 100 μl of the eluted volume was chromatographed on microparticulate reversed phase packing material with chemically bonded octadecyl groups on 5 μ silica using a high pressure pump and an electrochemical detector. The limit of detection for 5-hydroxytryptamine in this system is about 25 pg. Retinae from chicken and pigeon were analyzed individually whereas retinae from frog and goldfish were pooled 5 and 5.

RESULTS

Immunohistochemistry

Chicken. Numerous 5-hydroxytryptamine immunoreactive ovoid cell bodies were found in the innermost cell rows of the inner nuclear layer. They were generally situated two or three cell rows outwards in this layer and constituted approximately 5-10% of the cell bodies in their cell row. They sent processes to two sublayers of immunoreactive nerve fibres in sublaminae 1 and 4 in the inner plexiform layer. The innermost sublayer was the most prominent. The sublayers were connected by vertically running nerve fibres (Fig. 1). More rarely, cell bodies were seen in the innermost cell row of the inner nuclear layer. They were

more rounded and seemed to send processes only to the outermost of the two sublayers.

Ovoid cell bodies with weaker fluorescence were discerned in the middle of the inner nuclear layer and weakly fluorescent nerve fibres projected outwards and inwards from them. The fibres projecting inwards were only weakly fluorescent and therefore very hard to observe. The fibres projecting outwards reached the outer plexiform layer.

Pigeon. The number, shape and distribution of neurons displaying 5-hydroxytryptamine immunoreactivity in the pigeon retina resembled that found in chicken (Figs 2 and 3). However, the fluorescence intensity was much stronger which made it possible to distinguish the details of the neurons with their cell bodies in the middle of the inner nuclear layer. As in chicken they sent processes both inwards to the inner plexiform layer and outwards to the horizontal cells and the outer plexiform layer where they formed a layer of weakly fluorescent terminals (Fig 4).

Frog. Numerous 5-hydroxytryptamine immunoreactive cell bodies were found in the innermost several cell rows in the inner nuclear layer. Two different cell types could be distinguished in the innermost cell row: large and slightly polyhedral cells and more rounded, small cells, approximately of the size of the surrounding non-fluorescent perikarya in the same layer. The large polyhedral type occurred only in the innermost cell row of the inner nuclear layer whereas the other type was seen also a few cell rows outwards. The large type was slightly more common than the small. Both cell types sent processes to a dense plexus of nerve fibres evenly distributed

throughout the inner plexiform layer (Fig. 5). Short immunoreactive fibres were also seen encircling the ganglion cells and in the nerve fibre layer.

Goldfish. 5-hydroxytryptamine immunoreactive cell bodies constituted a few per cent of the cells in the innermost two cell rows in the inner nuclear layer. They were rounded when situated in the innermost cell row and more pear-shaped when situated further outwards. They sent processes to two sublayers of nerve fibres in sublaminae 1 and 5 in the inner plexiform layer and the two sublayers were connected by radially running nerve fibres. The outermost sublayer was somewhat more prominent than the innermost. At times a third, incomplete sublayer was seen in sublamina 3 (Fig. 6).

Cow, pig, cat, rabbit, guinea-pig, rat and mouse. No 5-hydroxytryptamine immunoreactivity was found in these species.

TABLE I

QUANTITATIVE ANALYSIS OF 5-HYDROXYTRYPTAMINE IN THE RETINA

Animal	No. of analyses	mean $\pm$ s.e.m.	
		ng/g wet weight	pmol/g wet weight
Chicken (6-8 weeks)	8	121.2 $\pm$ 7.02	687.8 $\pm$ 39.8
Pigeon (adult)	12	84.8 $\pm$ 10.7	481.2 $\pm$ 60.7
Frog (adult, 5 retinae per analysis)	4	1720 $\pm$ 420	9761 $\pm$ 2384
Goldfish (10-15 cm, 5 retinae per analysis)	6	1111 $\pm$ 146.4	6305 $\pm$ 831

Liquid-phase absorption tests. For testing the specificity, the 5-hydroxytryptamine antisera were incubated with a 30 mM solution of each of the following substances: 5-hydroxytryptamine, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, bufotenine, 6-hydroxytryptamine, 5-methoxytryptophan, 5-hydroxytryptophan, melatonin, L-tryptophan and norharmane. 5-hydroxytryptamine, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, and bufotenine blocked immunoreactivity in all species. Incubation with 6-hydroxytryptamine resulted in decreased immunofluorescence whereas incubation with the other substances did not affect fluorescence intensity or the distribution of fluorescent structures.

Quantitative analyses: High concentrations of 5-hydroxytryptamine were found in goldfish and frog and moderate concentrations were found in chicken and pigeon (see Table I). It is not likely that the high figures are caused by technical artefacts because simultaneous assays on rabbit retinae were in the expected range (48 ± 11 ng/g wet weight, four assays).

DISCUSSION

The antisera used in this study were from two sources, one of which was commercial. However, both sera were prepared in essentially the same way and the antibodies have been used in a number of studies on the distribution of 5-hydroxytryptamine in the brain of different vertebrates (Steinbusch 1981, Steinbusch et al. 1981). Their specificity was further tested by absorbing them with very high amounts of different indoleamines and related substances prior to testing them on histological sections. This gives a qualitative estimate of possible cross

reactivity under the circumstances the antibodies are used. Apart from 5-hydroxytryptamine four substances were found to react with the antibodies: 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, bufotenine and 6-hydroxytryptamine. Since, however, none of these are known from other assays to be present in the retina, it is not likely that they are responsible for the fluorescence seen in the immunohistochemical procedure. Five other related derivatives were ineffective in blocking the antibodies and are thus not likely to be demonstrable. The specificity of the antibodies is thus similar to the monoclonal one used by Osborne et al. (1982) which was raised to the same conjugate as the antisera used in this study.

In chicken and pigeon (Florén 1979) the indoleamine accumulating neurons constitute a subclass of amacrine cells. The cell bodies are usually situated in the innermost two or three cell rows of the inner nuclear layer and send processes that branch in two well developed sublayers in the inner plexiform layer. The distribution of the 5-hydroxytryptamine immunoreactive neurons is identical.

Indoleamine accumulating neurons have been observed also in frog retina (Ehinger, unpublished). These neurons have their cell bodies located in the innermost cell rows of the inner nuclear layer and their nerve fibres ramify throughout the inner plexiform layer in a manner that is identical to that of the 5-hydroxytryptamine immunoreactive neurons. The results agree very well with those of Osborne et al. (1981, 1982).

Indoleamine accumulating neurons have also been demonstrated in goldfish (Ehinger and Florén 1976). As seen in the electron microscope, they form a subclass of amacrine neurons

(Holmgren-Taylor 1983) with cell bodies in the innermost part of the inner nuclear layer and terminals distributed in two or three sublayers in the inner plexiform layer. The outermost of these sublayers is the best developed. The distribution of 5-hydroxytryptamine immunoreactive neurons seen in this study is identical, and there can be no doubt that the two methods demonstrate the same type of neurons. Osborne et al. (1982) have independently seen an identical distribution of 5-hydroxytryptamine neurons with a similar technique.

The complete agreement between the present immunohistochemical demonstration of endogenous 5-hydroxytryptamine and previous formaldehyde fluorescence histochemical or autoradiographical studies on retinae in which the neurons had accumulated an indoleamine (Ehinger and Florén 1976, Florén 1979, Osborne 1982b, Osborne et al 1982) shows the specificity of the immunohistochemical method. The antibodies used in this study have some degree of cross reactivity towards dopamine in test tube experiments (Steinbusch et al. 1978). However, the dopaminergic neurons are in the retina readily distinguishable from the indoleamine accumulating ones already on morphological grounds. No dopaminergic neurons were labelled in this study and in no other instance have such neurons been labelled with the antisera used in this work (Steinbusch, personal communication 1982). It follows that all cells demonstrated with the method not only contain 5-hydroxytryptamine but also have an uptake mechanism for indoleamines, a feature typical of neurons operating with a biogenic amine as transmitter (Iversen 1975). It is also evident that the 5-hydroxytryptamine found in the retina of cold-blooded vertebrates and birds is located in neurons which therefore are likely to use it as neurotransmitter.

A special kind of bipolar-like cells was observed already in formaldehyde histofluorescence studies on indoleamine uptake in the retina (Florén 1979). Typically they were less fluorescent than the indoleamine accumulating amacrine cells, suggesting that their uptake of indoleamines was less efficient than that of amacrine cells or, possibly, that whatever indoleamine they accumulated was transformed into some substance that would be less readily visible with formaldehyde histofluorescence. The present study has fully confirmed the presence of these cells. As in the previous study, they are characterized by being less fluorescent than amacrine cells, i. e. they are likely to contain less of the antigen or an antigen that is not identical with 5-hydroxytryptamine and which therefore reacts less well with the antibodies. It is at present not possible to distinguish between these alternatives. However, the indoleamine related substances which in the liquid-phase absorption tests did not influence the immunofluorescence (5-methoxytryptamine, 5-methoxytryptophan, 5-hydroxytryptophan, melatonin, L-tryptophan and norharmane) are unlikely as neurotransmitter candidates. It is worth noticing that Osborne et al. (1982), using a monoclonal antibody with a different sensitivity, did not see the bipolar-like cells. This also indicates that they have a lower antigen concentration or that it is not 5-hydroxytryptamine.

Since the bipolar-like cells seem to contain a substance similar to 5-hydroxytryptamine and also have an uptake mechanism for indoleamines it seems possible that they use an indoleamine as neurotransmitter. This is of considerable interest for two reasons. First, there have previously been no suggestions about the possible neurotransmitter in any bipolar cell type. Secondly, the bipolar-like cells show biochemical similarities with the 5-hydroxytryptamine containing amacrine cells but are never-

theless clearly distinguishable not only on morphological grounds but also in their biochemical characteristics seen in this work. There are thus at least two distinct types of indole-amine containing neurons in birds.

The bipolar-like cells have their cell bodies in the position of bipolar cells. The fibres projecting outwards reach the outer plexiform layer. However, the processes running inwards have much weaker fluorescence and are not regularly seen. This puts the direction of signal transmission in question for these cells, because usually the lowest transmitter content is found in dendritic processes which in this case then would be the ones in the innermost part of the inner nuclear layer. However, bipolar cells are not presumed to transmit centrifugally as the transmitter concentration thus would suggest. This is on the other hand what the interplexiform cells do (Ehinger 1982), but more evidence is necessary before it can be suggested that the bipolar-like cells are a morphological variant of interplexiform cells.

In retinae from guinea-pig, rat or mouse no indoleamine accumulating neurons have been observed in previous studies and these results are in this work confirmed by the lack of 5-hydroxytryptamine immunoreactive neurons. Similarly, Osborne et al. (1982) were unable to demonstrate any 5-hydroxytryptamine immunoreactive neurons in several mammals (rat, rabbit, cow, human). In cow (Osborne 1981, Ehinger et al. 1982) and pig (Ehinger et al. 1982) no formaldehyde histofluorescence could be seen. However, autoradiographic studies revealed an uptake of (^3H)-5-hydroxytryptamine in amacrine cells. The reason for the discrepancy has not been determined but it is possible that the 5-hydroxytryptamine taken up in these cells is rapidly transformed into some non-fluorescent metabolite. The absence of 5-

5-hydroxytryptamine immunoreactive neurons in these species tends to support such an hypothesis. In cat and rabbit indoleamine accumulating neurons have been observed in formaldehyde histo-fluorescence studies (Ehinger and Florén 1976). However, the content of 5-hydroxytryptamine is low in rabbit retina (see Table II) and the fact that no 5-hydroxytryptamine immunoreactive neurons are found in these species suggests that 5-hydroxytryptamine is not the neurotransmitter in their indoleamine accumulating neurons.

Previously published quantitative assays are summarized in Table II. It is worth noticing that Welsh (1964) found 5-hydroxytryptamine concentrations as high as up to 800 ng/g wet weight in frog retinae. This compares well with the present figures and also with those of Osborne (1982a) and Osborne et al. (1982). However, the range is rather wide in all analyses of frog retinae (see for example the high s.e.m. in the present study) suggesting considerable variations between individuals. Significant concentrations of 5-hydroxytryptamine occur also in fish retina, but again with rather wide differences between different analyses. There are several possible explanations for this but there are nevertheless no doubt significant amounts of 5-hydroxytryptamine in fish retina and, like in frogs, it is located in neurons.

The present study also confirms that there is a significant concentration of 5-hydroxytryptamine in the chicken retina (Suzuki et al. 1977, Florén and Hansson 1980, Osborne 1982a, Parkinson and Rando 1981) and adds very similar figures for the pigeon. The figures are lower than for the cold-blooded vertebrates.

TABLE II

Review of figures for 5-hydroxytryptamine in the retina(ng/g wet weight $\pm$ s.e.m. N.D. = not detectable)

	Bioassay, fluorimetry	HPLC, DANSYL chromatography, radioenzymatic assay
Cows	N.D. ¹⁾ , 100.3 $\pm$ 10.1 ⁴⁾	26.2 $\pm$ 7.2 ⁷⁾ , 31 $\pm$ 7 ⁹⁾ , 39 $\pm$ 4 ¹⁰⁾ , 15 $\pm$ 3 ¹²⁾
Pigs		5.9 $\pm$ 0.8 ⁷⁾
Normal rabbits	100-320 ¹⁾ , N.D. ²⁾	24 $\pm$ 5 ⁵⁾ , 36 $\pm$ 6 ⁶⁾ , 48 $\pm$ 11 ⁸⁾ , 30 $\pm$ 6 ⁹⁾ , 18 $\pm$ 3 ¹²⁾
Perfused rabbits		3.8 $\pm$ 0.9 ⁷⁾
Guinea-pigs		6.0 $\pm$ 0.6 ⁷⁾
Rats		9 $\pm$ 1 ¹²⁾
Chicken, adult	176 $\pm$ 12 ³⁾	34 $\pm$ 4 ⁵⁾ , 121.2 $\pm$ 7.02 ⁸⁾
Chicken, newborn		71 $\pm$ 5 ⁵⁾ , 90 $\pm$ 8 ⁶⁾ , 80 $\pm$ 8 ¹⁰⁾ , 119.4 $\pm$ 15.46 ¹¹⁾ , 127 $\pm$ 15 ¹³⁾
Chicken, newborn, dark adapted		70.3 $\pm$ 11.6 ¹¹⁾
Pigeon, adult		84.8 $\pm$ 10.7 ⁸⁾
Alligator	N.D. ¹⁾ (Alligator mississippiensis)	
Turtle	N.D. ¹⁾ (Chelydra serpentina)	
Lizard		96 $\pm$ 8 ¹⁰⁾ , 113 $\pm$ 29 ¹²⁾
Frog	100-800 ¹⁾	290 $\pm$ 18 ¹⁰⁾ , 500 $\pm$ 80 ¹⁰⁾ , 1720 $\pm$ 420 ⁸⁾ , 579 $\pm$ 79 ¹²⁾
Goldfish		1111 $\pm$ 146.4 ⁸⁾ , 231 $\pm$ 48 ¹²⁾
	(Carassius auratus)	
Alewife	200 ¹⁾ (Pomolabrus pseudoharengus)	
A catfish	300-600 ¹⁾ (Ameiurus nebulosus)	

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- 1). Welsh 1964, bioassay and fluorimetry
 - 2). Häggendal and Malmfors 1965, fluorimetry
 - 3). Suzuki, Noguchi, Miyake and Yagi 1977, fluorimetry
 - 4). Thomas and Redburn 1979, fluorimetry
 - 5). Florén and Hansson 1980, HPLC
 - 6). Florén and Hansson 1980, radioenzymatic method
 - 7). Ehinger, Hansson and Tornqvist 1981, HPLC
 - 8). This paper, HPLC
 - 9). Osborne 1980, HPLC
 - 10). Osborne 1982a, HPLC
 - 11). Parkinson and Rando 1981, HPLC
 - 12). Osborne, Nesselhut, Nicholas, Patel and Cuello 1982, HPLC
 - 13). Osborne, 1982b, HPLC

In summary, the indoleamine accumulating amacrine cells in chicken, pigeon, frog, and goldfish, thus contain endogenous 5-hydroxytryptamine as well as an uptake mechanism for it. Furthermore, high or moderate amounts of 5-hydroxytryptamine are found in retinae from these species while no or very low concentrations of endogenous 5-hydroxytryptamine can be found in the mammalian retina (Table II). These facts support the idea that 5-hydroxytryptamine is the neurotransmitter of the indoleamine accumulating neurons of cold-blooded animals and perhaps also of birds. They also confirm that the indoleamine accumulating neurons of mammals contain no or only very little 5-hydroxytryptamine and suggest that the content of 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine or bufotenine is also very low. The low 5-hydroxytryptamine content makes it an unlikely transmitter candidate in mammals. The transmitter of the mammalian indoleamine accumulating neurons thus remains elusive.

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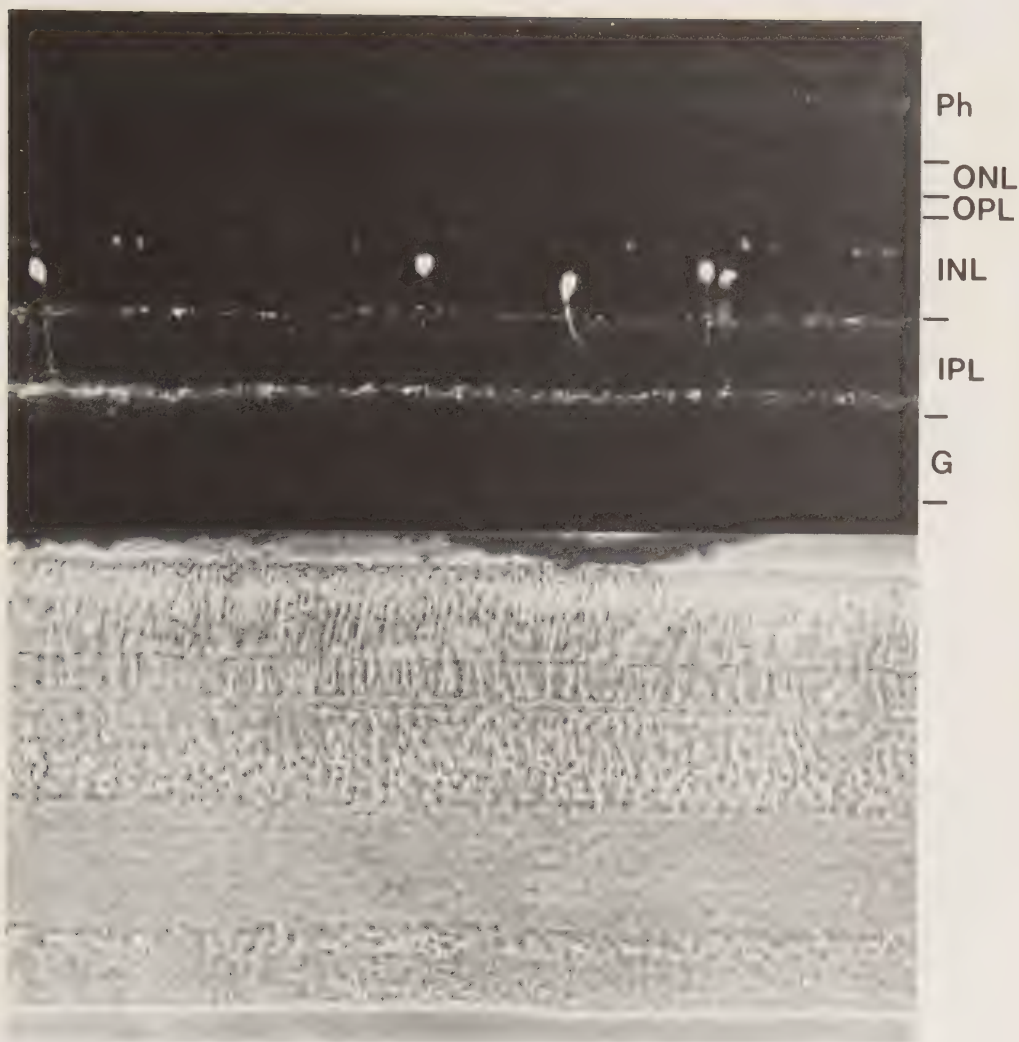


Fig. 1. 5-hydroxytryptamine immunoreactive neurons in the chicken retina. Cell bodies are situated in the innermost cell rows of the inner nuclear layer and send processes to nerve fibre layers in sublaminae 1 and 4 in the inner plexiform layer. Vertical nerve fibres connect these nerve fibre layers. Bipolar-like cell bodies are seen in the middle of the inner nuclear layer. Ph, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Above, fluorescence micrograph; below, phase contrast micrograph X 280.

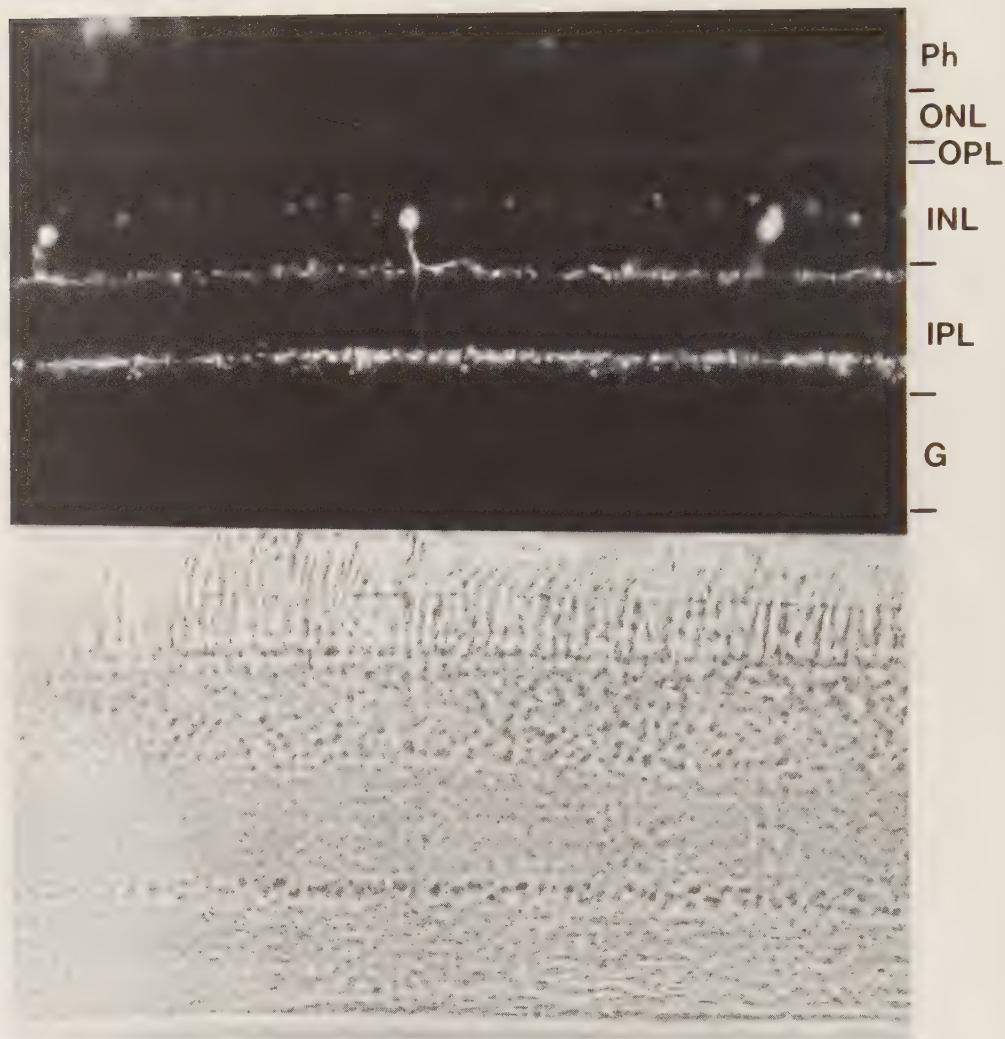


Fig. 2. 5-hydroxytryptamine immunoreactive neurons in pigeon retina. As in chicken cell bodies are found in the innermost cell rows of the inner nuclear layer sending processes to both nerve fibre layers in the inner plexiform layer. Bipolar-like cell bodies display weaker fluorescence and are found in the middle of the inner nuclear layer. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph X 280.



Fig. 3. 5-hydroxytryptamine immunoreactive neurons in pigeon retina. Cell bodies are rather frequent and localized to the innermost cell rows of the inner nuclear layer with processes forming two sublayers in the inner plexiform layer. Numerous bipolar-like immunoreactive cell bodies are seen in the middle of the inner plexiform layer. Designation of layers as in Fig. 1. Fluorescence micrograph X 180.

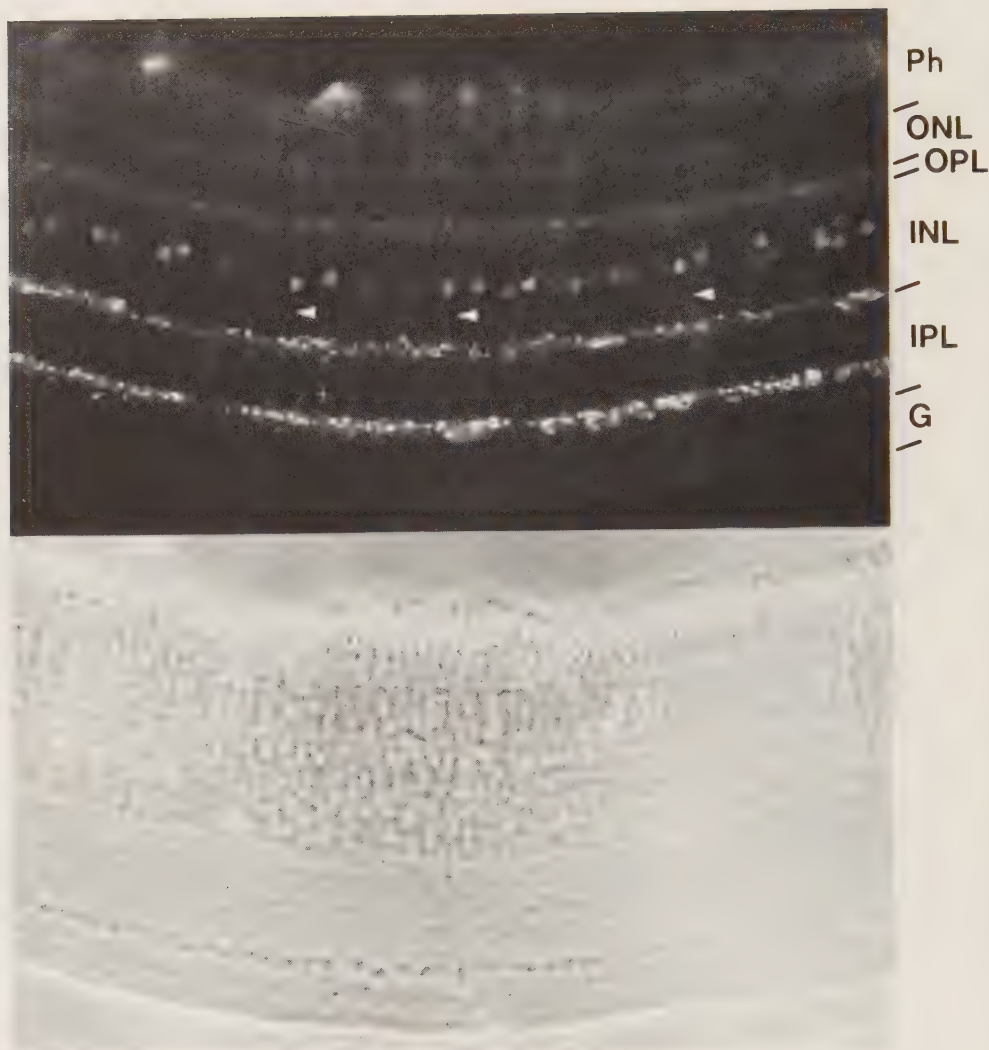


Fig. 4. 5-hydroxytryptamine immunoreactive bipolar-like cell bodies in the pigeon retina. These cell bodies are situated in the middle of the inner nuclear layer. They send weakly fluorescent processes inwards (arrow heads) to the inner plexiform layer and outwards to the outer plexiform layer and the horizontal cells where they form a layer of weakly fluorescent terminals. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph X 280.

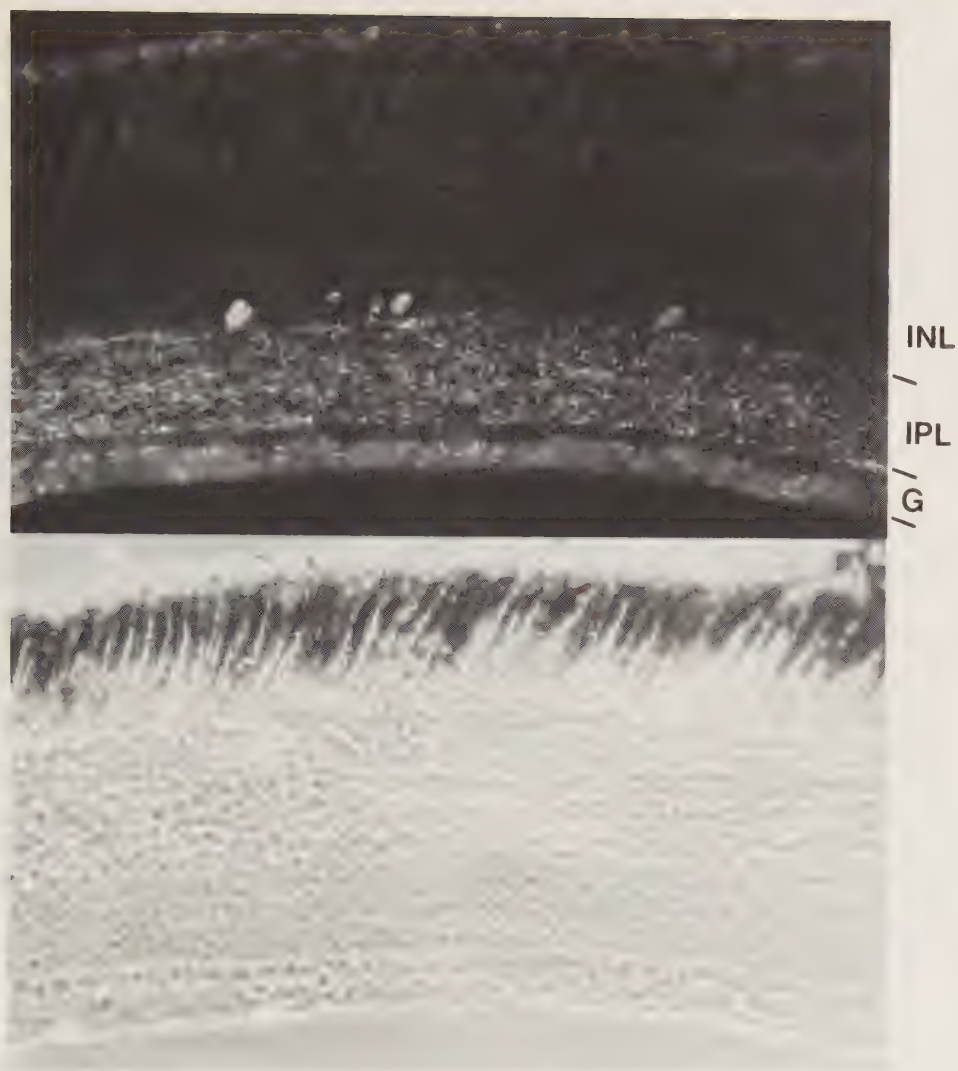


Fig. 5. 5-hydroxytryptamine immunoreactive neurons in frog retina. Two types of cell bodies are seen in the innermost cell rows of the inner nuclear layer. Their processes are evenly distributed throughout the inner plexiform layer. Immunoreactive nerve fibres are also seen encircling the ganglion cells and in the nerve fibre layer. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph X 280.

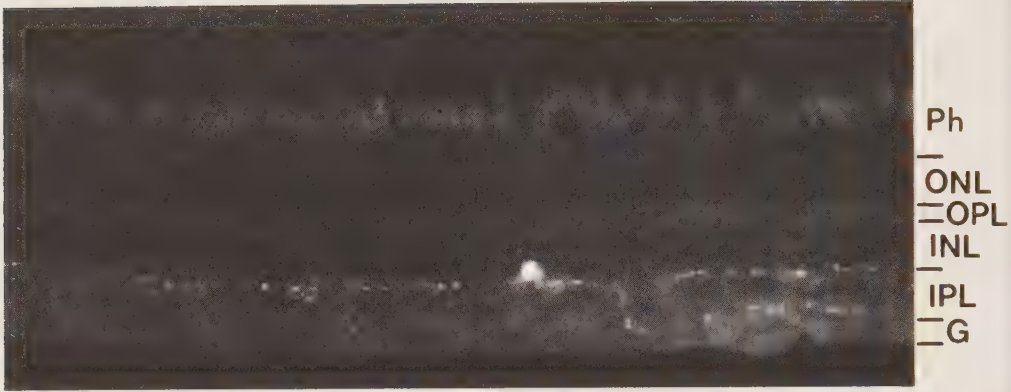


Fig. 6. 5-hydroxytryptamine immunoreactive neurons in goldfish retina. A rounded perikaryon is seen in the innermost cell row of the inner nuclear layer sending processes to two nerve fibre layers in sublaminae 1 and 5 of the inner plexiform layer. They are connected by radially running nerve fibres. Short twigs of immunoreactive fibres are also seen in sublamina 3. Designation of layers as in Fig. 1. Fluorescence micrograph X 280.

Peptide-containing Neurons in the Chicken Retina

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Four populations of cells showing immunoreactivity towards glucagon, somatostatin, enkephalin and neurotensin respectively were detected among the amacrine cells in the chicken retina. Processes of these different cell types formed sublayers in different laminae of the inner plexiform layer. The distribution of cell bodies and processes varied with the type of immunoreactive neuron investigated.

Key words: glucagon; somatostatin; enkephalin; neurotensin; amacrine cells; retinal neurons; chicken retina; immunocytochemistry.

1. Introduction

A number of retinal neurons – chiefly amacrine and horizontal cells – operate with well-known transmitters such as dopamine, GABA, glycine or acetylcholine (cf. Neal, 1976; Ehinger, 1976, 1978). However, these cells represent only a minor fraction of the total number of neurons in the retina, implying that there are several others with still unidentified transmitters. Recently, several peptides in the central and peripheral nervous systems have been ascribed neurotransmitter function (cf. Emson, 1979; Snyder and Innis, 1979; Hökfelt, 1980). Conceivably, peptides may play similar roles in the retina. In fact, recent studies, immunochemical as well as immunocytochemical, suggest that neuropeptides such as substance P, somatostatin, enkephalin, TRH and VIP occur in the retina (Brecha, Karten and Laverack, 1979; Humbert, Pradelles, Gros and Dray, 1979; Krisch and Leonhardt, 1979; Rorstad, Brownstein and Martin, 1979; Shapiro, Kronheim and Pimstone, 1979; Youngblood, Humm and Kizer, 1979; Karten and Brecha, 1980; Lorén, Tornqvist and Alumets, 1980; Martino et al., 1980; Yamada et al., 1980). We have therefore examined the retina for various neurohormonal peptides with the aid of immunocytochemistry. The occurrence of immunoreactivity suggesting the presence of several different peptides in different types of amacrine cells is described.

2. Materials and Methods

Retinae were obtained from ten White Leghorn chickens (14–20 weeks old). All tissue specimens included the central part of the retinal fundus; no effort was made to dissect other defined regions. Five chickens were killed by an overdose of diethyl ether. The remaining five chickens were anesthetized with Brietal®, perfused via the ascending aorta with 1 l of an ice-cold 4% formalin solution (40 g paraformaldehyde dissolved in 1 l 0.1 M-phosphate buffer: pH adjusted to 7.0 with 1 N-NaOH). The retinae were rapidly dissected out and stored in the fixative for 24–48 hr and rinsed for 24 hr in an ice-cold Tyrode buffer containing 5% sucrose. The specimens were frozen in dry ice and sectioned in a cryostat at –15°C. The sections (15 µm) were processed for the histochemical demonstration of various neuropeptides

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by the indirect immunofluorescence technique (Coons, Leduc and Connolly, 1955). The antisera used are listed in Table I. The site of antigen-antibody reaction was revealed with fluorescein-isothiocyanate-labelled goat anti-rabbit IgG (Statens Bakteriologiska Laboratorium, Stockholm, Sweden), used in dilution 1:20.

For controls, all antisera in the appropriate dilution were preincubated for 24 hr with an excess of the respective antigen* (10–100 $\mu\text{g}/\text{ml}$) and tissue sections were exposed to these inactivated sera. None of the cell bodies or processes described in Results showed up with the inactivated antisera. Each of the antisera used was tested for possible cross-reactivity with a number of peptides including glucagon, somatostatin, enkephalin and neurotensin (100 μg peptide per ml antiserum). No cross-reactivity could be demonstrated.

3. Results and Comments

Immunostaining was observed in several different types of amacrine cells but not in any other cell type. No fibres were observed in the outer plexiform layer, indicating that interplexiform cells do not react with the antisera. Amacrine cells are known to send out processes that ramify within various laminae of the inner plexiform layer, which is thick and exceptionally well developed in birds (Dubin, 1970; Yazulla, 1974). Five different laminae have been observed (Ramon y Cajal in Rodieck, 1973). Lamina 1 is the one closest to the inner nuclear layer, whereas lamina 5 is closest to the ganglion cells (Fig. 1).

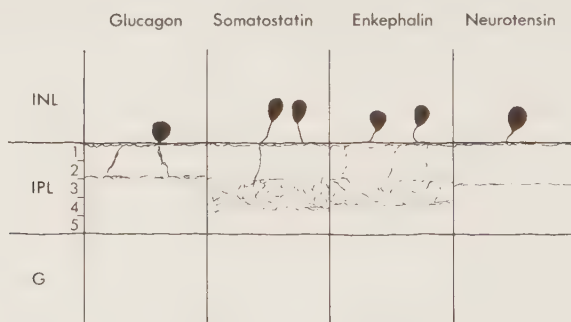


FIG. 1. Schematic morphology of peptide-containing neurons in the chicken retina. The cell bodies are situated in the inner nuclear layer (INL) with processes projecting into the inner plexiform layer (IPL) where they form sublayers in a pattern differing with the cell type. Note the differences in fibre density in sublaminae 3 and 4 between somatostatin and enkephalin. The drawing attempts to convey the actual difference. The photomicrographs in Figs 3–5 do not, because the antibodies used do not give the same staining intensity. G, ganglion cell layer.

Glucagon

Glucagon-immunoreactive cell bodies were observed in the innermost cell row of the inner nuclear layer (Fig. 2). They were rounded or slightly polyhedral and not appreciably different in size from the other cells in this row. Their number was small, at most representing only a few per cent of the total number of cells in this row. The cell bodies were seen to give off processes which divided dichotomously, forming a layer of intertwined varicose fibres in lamina 1 of the inner plexiform layer. Fine varicose fibres were seen to issue from this layer (rarely directly from a cell body) inwards through the inner plexiform layer to reach lamina 2, where they formed another

* Pure porcine glucagon was a kind gift from Dr Lise Heding, Novo, Copenhagen, Denmark. Synthetic somatostatin, enkephalin and neurotensin were purchased from UCB, Bruxelles, Belgium.

TABLE I
Source, working dilutions and characteristics of the peptide antisera used

Code	Antisera against	Antigen	Working dilution	References	Source
4304	Glucagon	Pure porcine pancreatic glucagon	1:40	Alumets et al. (1978a)	J. Holst, Dept. Clin. Chem. Bispebjerg Hospital, Copenhagen, Denmark
7811	Glucagon	Pure porcine pancreatic glucagon	1:80	Alumets et al. (1978a)	Å. Forsberg, Milab. Malmö, Sweden
19578	Somatostatin	Synthetic ovine somatostatin	1:40	Alumets et al. (1978a)	M. P. Dubois, Inst. Nat. Rech. Agr., Nouzilly, France
'leu-enk'	Enkephalin	Synthetic leu-enkephalin	1:60	Alumets et al. (1978b)	R. Y. Miller and K.-J. Chang, Wellcome Res. Lab. Burroughs Wellcome Co., Res. Triangle Park, N.J., USA
HC-8	Neurotensin	Synthetic bovine neurotensin	1:80	Sundler et al. (1977)	R. Carraway, Dept. Physiol. Harvard Med. Sch. Boston, Mass. USA

The glucagon antisera cross-react with gut type glucagon, and consequently do not require a free glucagon NH₂ terminus. The region specificity of the somatostatin antiserum is not known. The enkephalin antiserum cross-reacts poorly with met-enkephalin (Alumets et al. 1978b). The neurotensin antiserum is directed against the COOH-terminal six- to eight-amino-acid sequence (Carraway and Leeman, 1976).



FIG. 2. Glucagon immunofluorescence in chicken retina. Three fluorescent cell bodies can be seen in the innermost cell row of the inner nuclear layer (INL) with their processes forming two sublayers in laminae 1 and 2 of the inner plexiform layer (IPL). One cell can be seen to send processes to both sublayers. Fluorescence micrograph ($\times 330$).



FIG. 3. Somatostatin immunoreactive cell bodies in the innermost part of the inner nuclear layer (INL) with processes projecting into the inner plexiform layer (IPL) forming one layer of nerve fibres in lamina 1 and a wide plexus of nerve fibres in laminae 3 and 4. Fluorescence micrograph ($\times 300$).

narrow layer, slightly less dense than the one in lamina 1. The fibres issuing from the perikarya usually seemed to run for some distance in lamina 1 before branching into lamina 2.

Somatostatin

Numerous somatostatin-immunoreactive, pear-shaped cell bodies were observed in the inner nuclear layer, usually located one or two cell rows outwards from the inner plexiform layer (Fig. 3). The cells were in most cases located distally to the glucagon immunoreactive cell bodies, and about as far out as amacrine cell bodies can be found in birds (cf. Cajal in Rodieck, 1973). These cells gave off processes which ran inwards about as far as to lamina 1 of the inner plexiform layer, where they branched, forming a narrow layer of fine varicose fibres. Other branches of the same cells were seen to proceed radially further inwards to the inner plexiform layer where they branched again, forming a loosely tiered, wide plexus of fine varicose fibres throughout laminae 3 and 4 (Fig. 4). At times, three sublayers of immunoreactive fibres could be recognized in this plexus, but this sublayering was never very conspicuous. The innermost lamina was devoid of immunoreactive terminals.



FIG. 4. Somatostatin immunoreactivity in chicken retina. The cell bodies are situated in the inner nuclear layer (INL) giving off processes that form one layer of nerve fibres in lamina 1 of the inner plexiform layer (IPL) while other processes proceed radially further inwards forming a loosely tiered plexus in laminae 3 and 4. Note that several cells seem to send processes to both sublayers. Fluorescence micrograph ($\times 400$).

Enkephalin

A large number of pear-shaped enkephalin-immunoreactive cell bodies were seen in the innermost part of the inner nuclear layer. The cell bodies were situated one, two and sometimes even three cell rows distally within this layer (Fig. 5). Processes extended from the cell bodies radially into the inner plexiform layer where they formed



FIG. 5. Enkephalin immunoreactive neurons in chicken retina. The cell bodies are located in the inner nuclear layer (INL) and their processes form one layer in lamina 1 of the inner plexiform layer (IPL) and another in laminae 3 and 4. Fluorescence micrograph ($\times 360$).



FIG. 6. Neurotensin immunoreactive cell body in the innermost part of the inner nuclear layer (INL). Processes are seen to project inwards to form two sublayers in laminae 1 and 3 of the inner plexiform layer, well discernible in the microscope. They did not photograph well because the fluorescence was weak and faded rapidly upon illumination ($\times 320$).

one layer of fine varicose fibres in lamina 1 and a second, broad and much less defined layer in laminae 3 and 4. At times three evenly dispersed sublayers could be discerned within this plexus.

Neurotensin

Neurotensin-immunoreactive, pear-shaped cell bodies were located in the innermost part of the inner nuclear layer. Most of them were situated two or three cell rows away from the inner plexiform layer, but some were contiguous with it (Fig. 6). The neurotensin cells were fewer than the ones containing immunoreactive glucagon, somatostatin or enkephalin. Each cell body was seen to send a fine process towards the inner plexiform layer. These processes started to divide at lamina 1 of the inner plexiform layer, where they formed a narrow sublayer of fine varicose fibres. They were also seen to branch to form another sublayer of fine varicose fibres in lamina 3. Both these sublayers appeared less dense than the ones formed by glucagon, somatostatin or enkephalin fibres. The immunofluorescence of the processes was very weak and did not photograph well.

4. Discussion

The antisera of the present study have all previously been used to establish the cellular localization of glucagon, somatostatin, enkephalin and neurotensin respectively in tissues of the chicken (see Table I).

The glucagon, somatostatin, enkephalin and neurotensin immunoreactive cell bodies of the chicken retina were invariably found in the inner part of the inner nuclear layer, giving off processes that reached one or more laminae of the inner plexiform layer. This distribution pattern fits well with that of amacrine cells. The processes of the amacrine cells may reach one or two, rarely more, of the five laminae recognized in the inner plexiform layer in the avian retina, or they may be diffusely distributed without any definite sublayering (Cajal in Rodieck, 1973; Yazulla, 1974). The immunoreactive cells observed in this study were mostly stratified, but the pattern of stratification differed with the cell type studied. In the present study processes of immunoreactive amacrine cells were seen in two or more laminae in the inner plexiform layer. It cannot be excluded that the multistratified cells in reality represent several monostratified cells superimposed upon each other when viewed in the microscope. However, this is probably not the case because many immunoreactive cells were actually seen to send their processes to more than one layer.

'Amacrine cells of association' is a special cell type which is common in birds only (Cajal in Rodieck, 1973) and which is of some special interest because it projects widely and receives input from centrifugal fibres. The cell bodies are situated high in the inner nuclear layer like the somatostatin, enkephalin and neurotensin immunoreactive cell bodies. There is thus a possibility that at least some of the immunoreactive cells belong to this special group of amacrine cells.

In birds amacrine neurons working with transmitters such as GABA, glycine, dopamine, acetylcholine and an indolamine have previously been identified. The GABA neurons (identified by their ability to accumulate ^3H -GABA) occur among the horizontal and amacrine cells (Marshall and Voaden, 1974a). Most of the terminals occur in lamina 5 and many cell bodies are found in the innermost cell row of the inner nuclear layer. Glycine neurons (identified by their ability to accumulate ^3H -glycine) occur among amacrine cells but do not seem to be stratified (Marshall and Voaden,

1974b). Dopaminergic neurons (identified by fluorescence histochemistry) which occur in the innermost cell row of the inner nuclear layer are rare, but form a dense fibre network in lamina 1 and less extensive ones in laminae 3 and 5 (Ehinger, 1967, Stoeckel et al., 1976). Indolamine neurons (identified by fluorescence histochemistry) have their perikarya in the inner third of the inner nuclear layer and their processes in laminae 1, 4, and 5 of the inner plexiform layer (Florén, 1979). Cholinergic neurons (identified by acetylcholinesterase staining and by their ability to accumulate ^3H -choline) have terminals most abundant in laminae 2 and 4 of the inner plexiform layer; most of their cell bodies occur in the innermost cell row of the inner nuclear layer (Nichols and Koelle, 1968; Baughman and Bader, 1977).

Thus, neither of the cell types operating with GABA, glycine, dopamine, indolamine or acetylcholine has a morphology that fits with that of the immunoreactive cells seen in the present study. We cannot exclude that subpopulations of immunoreactive cells are identical with subpopulations of the other cell types, although it appears most likely that the neuropeptides and the conventional neurotransmitters are located in different cells.

The immunocytochemical distribution of somatostatin and enkephalin has previously been described by Krisch and Leonhardt (1979), Yamada et al. (1980) and Brecha et al. (1979) in the retina of the rat, goldfish and pigeon, respectively. The present results are in good agreement. Of the peptides demonstrated in this study immunoreactive neurotensin and glucagon has previously not been reported in the retina, although they have been found in the brain (Carraway and Leeman, 1973, 1976; Conlon, Sampson, Dobbs, Orci and Unger, 1979; Lorén, Alumets, Håkanson, Sundler and Thorell, 1979). Here, immunoreactive glucagon seems to represent gut type rather than pancreatic type glucagon (Conlon et al., 1979; Lorén et al., 1979). The exact chemical identity of the immunoreactive peptides demonstrated in the retina has to be established by detailed chemical and other analyses.

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Somatostatin and VIP Neurons in the Retina of Different Species

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Summary. Neurons displaying somatostatin or vasoactive intestinal polypeptide (VIP) immunoreactivity were detected among the amacrine cells in the retina of baboon, cynomolgus monkey, squirrel monkey, cow, pig, cat, rabbit, guinea-pig, rat, mouse, frog and goldfish. Generally, immunoreactive cell bodies were located in the inner nuclear layer with processes ramifying in three more or less well-defined sublayers in the inner plexiform layer. The density of the sublayers and their location varied with the peptide and species investigated. In most cases there was a sublayer in the outermost part (Ramon y Cajal's sublamina 1) of the inner plexiform layer and this sublayer was usually the best developed. In some species a few somatostatin fibres were also detected in the outer plexiform layer, suggesting that some interplexiform cells contain somatostatin. In the baboon VIP was found exclusively in interstitial amacrine cells which have their cell bodies and processes entirely within the inner plexiform layer.

Introduction

The retina, being an extension of the brain, comprises several different neuronal systems which produce and store neurotransmitters such as dopamine, gamma-aminobutyric acid (GABA), glycine and acetylcholine. However, the neurotransmitters of many retinal neurons remain unidentified. A number of peptides, such as VIP (vasoactive intestinal polypeptide), substance P, enkephalin, somatostatin, neurotensin and glucagon have emerged as neurotransmitter candidates in the central and peripheral nervous systems (Hökfelt et al. 1980; Håkanson et al. 1981). Recently, immunochemical and immunohistochemical studies have demonstrated neuropeptides also in the

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retina of a few species (Brecha et al. 1979; Humbert et al. 1979; Krisch and Leonhardt 1979; Rorstad et al. 1979; Shapiro et al. 1979; Eskay et al. 1980; Karten and Brecha 1980; Lorén et al. 1980; Yamada et al. 1980; Brecha et al. 1981; Buckerfield et al. 1981; Eriksen and Larsson 1981; Fukuda et al. 1981; Osborne et al. 1981; Tornqvist et al. 1981; Unger et al. 1981; Karten and Brecha 1982). Thus, both somatostatin and VIP have been observed in the retina of a few species in previous studies. However, the occurrence and distribution of neurons storing catecholamines or amino acids as neurotransmitters varies widely between different species (Ehinger 1982). As this may also be the case with peptide containing neurons we have done a comparative study on the occurrence and distribution of somatostatin and VIP immunoreactive neurons in a number of different species.

Materials and Methods

Retinae were obtained from the following animals: baboon (*Papio papio*), cow, pig, cat, rabbit, guinea-pig, rat, mouse, goldfish (*Carassius auratus*) and frog (*Rana temporaria*), at least four of each species. In addition specimens were obtained from one cynomolgus monkey (*Macaca fascicularis*) and one squirrel monkey (*Saguineus oedipus*). Monkeys and cats were killed by exsanguination under pentobarbital anaesthesia. Retinae from cows and pigs were obtained from a nearby slaughter-house. Two pigs, which received intraocular injections of colchicin, 50 µg and 500 µg, respectively, were killed with pentobarbital sodium 6 or 24 h after injection. The rabbits were killed by air embolization, guinea-pigs, rats and mice by an overdose of diethyl ether, goldfish and frogs by decapitation. The retinae were rapidly dissected out and fixed by immersion in ice-cold 4% formaldehyde solution (40 g paraformaldehyde dissolved in 1 l 0.1 M phosphate buffer; pH 7.2) for 24 h and rinsed for 24–28 h in an ice-cold Tyrode buffer containing 5% or 30% sucrose. The specimens were then frozen on dry ice and sectioned in a cryostat at -20°C . In addition, retinae of cats, rabbits, guinea-pigs, rats and mice were stretched on microscope slides, fixed in a solution of formaldehyde and picric acid, dehydrated, cleared in xylene and hydrated in a series of ethanol solutions (Costa et al. 1980). The cryostat sections (15 µ) and whole mount preparations were processed for the immunohistochemical demonstration of somatostatin or VIP using the indirect immunofluorescence technique (Coons

Fig. 1. VIP-immunoreactivity in the baboon retina. Immunoreactive nerve fibres situated in the middle of the inner plexiform layer connect rounded or slightly ovoid VIP cell bodies also situated in the middle of the inner plexiform layer. The photoreceptors are weakly autofluorescent: *Ph*, photoreceptors; *INL*, innernuclearlayer; *IPL*, innerplexiformlayer; *G*, ganglioncell layer. Fluorescence micrograph $\times 375$

Fig. 2. VIP cell bodies in the cow retina. The cell bodies are rounded or slightly polyhedral and send processes to two or three nerve fibre layers in the inner plexiform layer. Fluorescence micrographs $\times 280$

Fig. 3. VIP nerve fibres in the cow retina forming sublayers in sublamina 1 and 5 of the inner plexiform layer. The incomplete sublayer at times seen between them is not shown in figure. In all species studied the VIP fibres are coarser than the somatostatin fibres. There is some non-specific autofluorescence in the photoreceptors. Designation of layers as in Fig. 1. Fluorescence micrograph $\times 280$

Fig. 4. Whole mount preparation from guinea-pig retina showing rather coarse, varicose VIP fibres forming a dense plexus. Fluorescence micrograph $\times 240$





G IPL INL

Fig. 5. VIP fibres in the guinea-pig retina. Only two of the three VIP fibre sublayers is readily seen in the figure. The outermost is denser than the sublayer situated further inwards. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph $\times 280$

Fig. 6. VIP in the guinea-pig retina. A rounded or somewhat pearshaped cell body is situated in the innermost cell row of the inner nuclear layer sending processes to a nerve fibre sublayer situated in the outermost part of the inner plexiform layer. The sublayers in sublaminae 3 and 5 are not well developed in this particular section. Designation of layers as in Fig. 1. left, fluorescence micrograph; right, phase contrast micrograph $\times 280$

et al. 1955). Two different somatostatin antisera were used. One (code no 19578, a kind gift from Dr. M.P. Dubois, Nouzilly, France) was used in dilution 1:80. It has previously been successfully used for demonstrating structures known to contain somatostatin (Lundqvist et al. 1979; Alumets et al. 1980). The other somatostatin antiserum (code no 31100) was bought from Immuno Nuclear Corporation, Stillwater, Minnesota, USA, and used in dilution 1:80. Both somatostatin antisera gave identical results. For the demonstration of VIP immunoreactivity a rabbit antiserum (code no 7852, Milab, Malmö, Sweden) raised against highly purified porcine VIP was used in dilution 1:320. The antiserum has been successfully used in several previous immunohistochemical studies (Sporrong et al. 1982; Uddman et al. 1982). The site of antigen-antibody reaction was revealed with fluorescein-isothiocyanate labelled goat antirabbit Ig G (Statens Bakteriologiska Laboratorium, Stockholm, Sweden and Dako, Copenhagen, Denmark) in dilution 1:20. Some of the whole mounts were subsequently washed, incubated with PAP complex and stained for peroxidase (Sternberger 1979). For controls all antisera in the appropriate dilution were preincubated for 24 h with an excess of the respective antigen (10–100 µg/ml) and tissue sections were exposed to these inactivated antisera. The controls were negative. There was at times some non-specific autofluorescence in the photoreceptor outer segments.

Cross reactivity with unknown peptides containing immunoreactive amino acid sequences recognized by the different antibodies cannot be excluded. Therefore it would be appropriate to call the immunoreactive material VIP-like or somatostatin-like. However, for brevity, the immunoreactive nerve fibres are referred to as somatostatin or VIP fibres in the text.

Results

Baboon

In the baboon retina, delicate, varicose somatostatin fibres were seen as a thin layer in the outermost part and in the innermost half of the inner plexiform layer. A third, incomplete, nerve fibre layer was seen in the middle of the inner plexiform layer. Fibres connecting the sublayers were not observed nor were any immunoreactive cell bodies seen (Fig. 12).

Varicose VIP fibres were found in a single layer in the middle of the inner plexiform layer. They were rather coarse and issued from large, rounded or slightly ovoid VIP cell bodies also situated in the middle of the inner plexiform layer (Fig. 1).

Cynomolgus Monkey

Somatostatin fibres were found in a well defined sublayer in the outer part of the inner plexiform layer and in a less well defined sublayer in the middle of the same layer. The sublayers were connected by fine varicose nerve fibres (Fig. 12). Scattered VIP nerve fibres were seen in the middle of the inner plexiform layer (Fig. 13). Cell bodies displaying somatostatin or VIP immunoreactivity were not seen.

Squirrel Monkey

Somatostatin fibres formed three sublayers in the inner plexiform layer (Fig. 12). A rather dense sublayer was situated at the border to the inner

nuclear layer. In the middle and in the innermost part of the inner plexiform layer less dense sublayers were situated. Sparsely occurring, rounded somatostatin cell bodies were found in the innermost cell row in the inner nuclear layer.

The distribution of VIP fibres was similar to the somatostatin fibres with three sublayers of nerve fibres in the inner plexiform layer. Sparsely occurring, rounded VIP cell bodies were situated in the innermost cell row of the inner nuclear layer (Fig. 13).

Cow

Somatostatin fibres formed a continuous nerve fibre sublayer in the outermost part of the inner plexiform layer. At times scattered varicose nerve fibres could be seen further inwards forming two incomplete nerve fibre sublayers in the middle and innermost part of the inner plexiform layer. In some cases very delicate, varicose nerve fibres were seen to run outwards through the inner nuclear layer joining short nerve fibres situated at the border between the inner nuclear and outer plexiform layer. Somatostatin cell bodies were not observed (Fig. 12).

Sparse, rounded or slightly polyhedral VIP cell bodies were found in the innermost cell row in the inner nuclear layer. They issued processes forming two sublayers situated in sublaminae 1 and 5 of the inner plexiform layer. A third, less well defined sublayer of nerve fibres was seen between these. The three nerve fibre sublayers were at times connected by vertically running nerve fibres. Single VIP cell bodies could be seen to send processes to all sublayers in the inner plexiform layer. As in all other species studied, the VIP fibres were coarser than the somatostatin fibres, particularly the few tens of microns next to the perikaryon (Figs. 2 and 3).

Pig

Varicose somatostatin fibres were found in the inner plexiform layer forming three sublayers. One was situated in the innermost part of the inner plexiform layer. In the middle of this layer a less well defined sublayer was noted, while a very dense sublayer was found at the border to the inner

Fig. 7. Whole mount preparation from rat retina. A dense network of beaded VIP-fibres is seen. Fluorescence micrograph $\times 325$

Fig. 8. Somatostatin fibres in the cat retina. Besides the three nerve fibre sublayers in the inner plexiform layer varicose fibres are seen to run outwards through the inner nuclear layer joining short nerve fibres situated in the outer plexiform layer. Designation of layers as in Fig. 1. Fluorescence micrograph $\times 280$

Fig. 9. Somatostatin fibres in the goldfish retina forming a well defined sublayer in the outermost part of the inner plexiform layer and two less well defined sublayers further inwards in this layer. A rounded cell body is situated in the innermost cell row of the inner nuclear layer. Designation of layers as in Fig. 1. Fluorescence micrograph $\times 280$





10



11



nuclear layer. At times fine, varicose nerve fibres were seen in the outermost part of the inner nuclear layer (Fig. 10). Extremely sparsely occurring, ovoid somatostatin containing cell bodies were seen in the innermost cell rows of the inner nuclear layer. Intraocular injection of colchicin did not result in an increased number of somatostatin cell bodies.

VIP fibres formed two layers of very weakly fluorescent varicose nerve fibres in the outermost half of the inner plexiform layer (Fig. 13). Weakly fluorescent, very sparsely occurring VIP cell bodies were seen in the innermost cell row in the inner nuclear layer.

Cat

Fine, varicose somatostatin fibres formed well developed sublayers in the outermost, middle and innermost parts of the inner plexiform layer. The outermost nerve fibre layer was very dense, the innermost was less dense but still continuous and the one in the middle discontinuous (Fig. 11). Obliquely running nerve fibres were seen to connect these three sublayers. At times fine varicose fibres could also be seen running outwards through the inner nuclear layer joining short nerve fibres situated at the border between the inner nuclear and outer plexiform layers (Fig. 8). Whole mount preparations displayed a rather thin network of fine varicose fibres. Somatostatin cell bodies were not found nor were any VIP fibres or cell bodies discerned.

Rabbit

Fine, weakly fluorescent varicose somatostatin fibres were found in the outermost part of the inner plexiform layer (Fig. 12). At times fine varicose nerve fibres were seen further inwards, in the innermost part of the inner plexiform layer. A fine mesh-work of somatostatin fibres was seen in whole mount preparations. Somatostatin cell bodies were not found.

The distribution of VIP fibres (Fig. 13) was similar to that of the somatostatin fibres with a sublayer of fine, weakly fluorescent varicose nerve fibres in the outermost part of the inner plexiform layer and sparsely occurring fibres further inwards in the inner plexiform layer. A thin network

Fig. 10. Somatostatin fibres in the pig retina forming three sublayers in the inner plexiform layer. The one in the middle is less well defined than the other two situated in sublaminae 1 and 5 of the inner plexiform layer. The sublayer in sublamina 1 is usually better developed than shown in the figure. Furthermore, a somatostatin fibre is seen in the outer plexiform layer. *Ph*, photoreceptors; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *G*, ganglion cell layer. Above, fluorescence micrograph; below, phase contrast micrograph $\times 280$

Fig. 11. Somatostatin fibres in the cat retina forming three sublayers in the inner plexiform layer. The one in the middle is discontinuous and less well developed than the sublayers situated in the outermost and innermost parts of the inner plexiform layer. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph $\times 180$

of varicose fibres was seen in whole mount preparations. VIP cell bodies were not found.

Guinea-Pig

A continuous layer of fine and weakly fluorescent varicose somatostatin fibres was seen in the outermost part of the inner plexiform layer. Twigs of somatostatin fibres were also seen further inwards in the middle and innermost parts of the inner plexiform layer (Fig. 12). Whole mount preparations displayed a sparse network of beaded fibres. Somatostatin cell bodies were not seen.

VIP fibres formed three sublayers in the inner plexiform layer. The outermost was continuous and denser than the other two which were discontinuous and situated respectively in the middle and innermost parts of the inner plexiform layer (Fig. 5). Radially running nerve fibres connected these three nerve fibre layers. In whole mount preparations a dense plexus of varicose interlacing nerve fibres was seen (Fig. 4). The VIP cell bodies were pear-shaped, sparsely occurring and situated in the innermost cell row of the inner nuclear layer (Fig. 6).

Rat

Fine, varicose somatostatin fibres were found in sublaminae 1, 3 and 5 of the inner plexiform layer. The outermost sublayer was dense and continuous while the others were discontinuous. Occasionally radial somatostatin fibres were seen in the inner nuclear layer joining short horizontal twigs in the outermost part of the same layer (Fig. 12). Cell bodies were not detected.

VIP fibres formed two layers, one in the outermost part of the inner plexiform layer with processes projecting inwards reaching another nerve fibre layer situated in the innermost part of the inner plexiform layer (Fig. 13). In whole mount preparations a very dense network of beaded VIP fibres was seen (Fig. 7). Sparsely occurring, pear-shaped cell bodies were found in the innermost cell row of the inner nuclear layer. Their size and shape were similar to most other cells in this row.

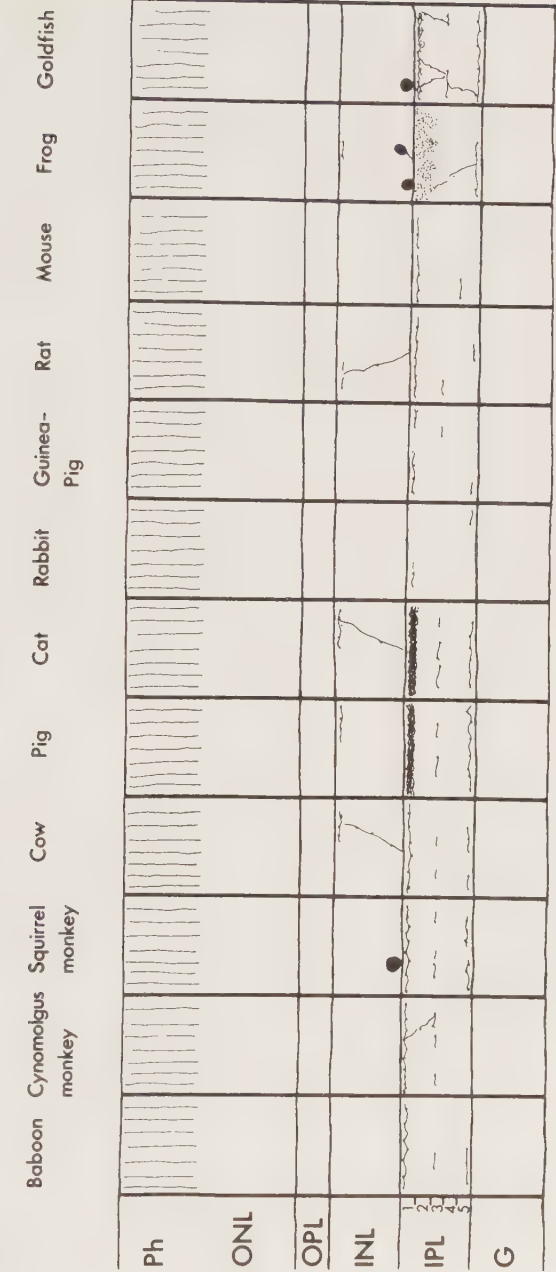
Mouse

Extremely fine, varicose somatostatin fibres formed two sublayers of different density in the inner plexiform layer (Fig. 12). The denser was situated in the outermost part of the inner plexiform layer while the looser was found in the innermost half of this layer. In whole mounts a network of fine fibres were seen. Somatostatin cell bodies were not seen.

Frog (Rana temporaria)

Somatostatin fibres formed a loose plexus in the outermost third of the inner plexiform layer. Such fibres were also seen at the border to the ganglion cell layer. Perpendicular fibres connected these nerve fibre layers. Short

Fig. 12. Schematic drawing illustrating the morphology of somatostatin neurons in the species investigated. Fibres usually form two or three more or less well defined sublayers in the inner plexiform layer. Cell bodies are ovoid or slightly pearshaped and situated in the innermost part of the inner nuclear layer. Interplexiform somatostatin-immunoreactive fibres are found in cow, pig, cat, rat and frog.



twigs of somatostatin fibres were occasionally discerned at the border between the inner nuclear and outer plexiform layers. Rounded cell bodies were found in the innermost cell rows in the inner nuclear layer (Fig. 12). Weakly fluorescent pear-shaped cell bodies were also frequently discerned a few cell rows outwards in the inner nuclear layer.

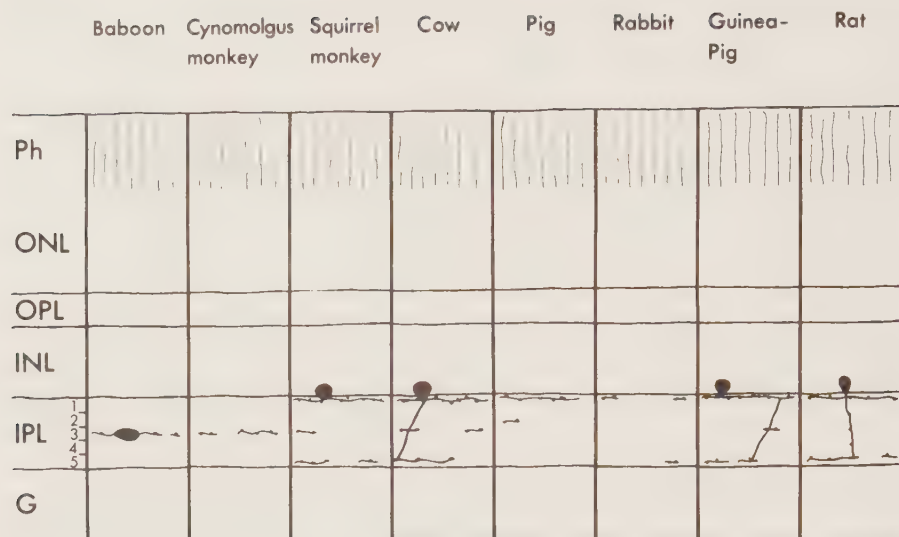


Fig. 13. Schematic drawing illustrating VIP neurons in the species investigated. VIP fibres are coarser than the somatostatin fibres. The VIP cell bodies are more frequently occurring and usually somewhat larger than the somatostatin ones. They are rounded or slightly polyhedral. In the baboon retina VIP-neurons are found exclusively in the middle of the inner plexiform layer constituting interstitial amacrine cells. Designation of layers as in Fig. 10

Goldfish

A dense layer of somatostatin nerve fibres was found in the outermost part of the inner plexiform layer. In addition, less well defined layers of nerve fibres were found in the middle and in the innermost part of the inner plexiform layer. Vertically running nerve fibres connected these layers but were not seen to run directly from the outermost to the innermost sublayer. Somatostatin cell bodies were found in the innermost cell row in the inner nuclear layer. The cell bodies were rounded or slightly pear-shaped (Fig. 9). No somatostatin ganglion cells have been found. A few such cells have been reported by Yamada et al. (1980) but because of their scarcity the difference in results may be due to random variations. VIP cell bodies or nerve fibres were not seen in this study.

Discussion

Somatostatin has been isolated, characterized and quantitated in the retina of chicken, pigeon, rat and man (Rorstad et al. 1979; Shapiro et al. 1979; Lake and Patel 1980; Rorstad et al. 1980; Yamada et al. 1980; Brecha et al. 1981; Buckerfield et al. 1981). In addition, immunohistochemical studies have demonstrated somatostatin in the retina of goldfish, frog, chicken and pigeon, but only in one mammal, the rat (Krisch and Leonhardt 1979; Yamada et al. 1980; Brecha et al. 1981; Buckerfield et al. 1981; Eriksen and Larsson 1981; Tornqvist et al. 1981). In all species the somatostatin

immunoreactivity was found in amacrine cells with processes restricted to three more or less well defined sublayers in the inner plexiform layer. VIP neurons have so far been reported only in the rat and pigeon (Lorén et al. 1980; Eriksen and Larsson 1981; Karten and Brecha 1982), in amacrine cells with their cell bodies located in the innermost third of the inner nuclear layer and with their processes restricted to two sublayers in the inner plexiform layer. Both somatostatin and VIP neurons have thus always been found to be stratified (usually multistratified) varieties of amacrine cells.

The present study on twelve different species, ten of which are mammalian, shows that both somatostatin and VIP immunoreactive peptides are ubiquitous components also of the mammalian retina. Somatostatin processes are usually delicate and varicose. They emanate from the perikaryon without any well marked axon hillock, and form sublayers in the inner plexiform layer. The outermost one (sublamina 1 in Ramon y Cajal's notation) usually has the highest fibre density, but species differences are frequent (Fig. 12). The somatostatin fibres are less readily demonstrated in some species than in others. In particular, they are weakly immunoreactive and hard to demonstrate in rabbits and guinea-pigs and in these species cell bodies have not been seen. In chicken and pigeon, on the other hand, somatostatin nerve fibres and cell bodies abound (Brecha et al. 1981; Buckerfield et al. 1981; Tornqvist et al. 1981; Karten and Brecha 1982). This agrees well with the assays published by Eskay et al. (1980): they found only 7 ± 0.6 pg/mg in rabbits and 21 ± 1.9 pg/mg in guinea-pigs, but more in cows (49 ± 8.2 pg/mg) and most in chicken (102 ± 8.2 pg/mg).

VIP fibres have a distribution that superficially resembles that of the somatostatin fibres. However, in several species the details distinguish the two (Figs. 12 and 13). VIP fibres are usually coarser than the somatostatin ones and their cell bodies are more numerous and also usually somewhat larger, rounded and at times polyhedral whereas the somatostatin perikarya are ovoid or pearshaped with their long axis directed radially. Further, in for instance guinea-pigs, the somatostatin fibres form a thin sublayer in sublamina 1 of the inner plexiform layer whereas the VIP fibres form three distinct sublayers. In cynomolgus monkeys and baboons the VIP fibres are restricted to a single sublayer in the middle of the inner plexiform layer whereas the somatostatin fibres occur in three sublayers in different parts of the inner plexiform layer. It is thus clear that even though the fibres distribute similarly they vary independently. The similarity in fibre distribution therefore cannot be taken as evidence for any co-occurrence of the two peptides within the same neuron. Correspondingly, a similar but not identical distribution has been seen for neurotensin and somatostatin fibres in the pigeon retina (Brecha et al. 1981).

Neuropeptides have in the retina so far usually been localized to amacrine cells and the present species survey confirms that this is indeed the case with somatostatin and VIP. However, it is important to notice that there are exceptions, two of which are reported here for the first time: VIP neurons localized exclusively within the inner plexiform layer and somatostatin fibres in the outer plexiform layer. Neurons entirely localized within

the inner plexiform layer are known also from metal impregnation studies (Ramon y Cajal in Rodieck 1973) and have traditionally been regarded as accidentally displaced amacrine cells. However, the present observation of a special histochemical marker in certain of them suggest that their location is not an accident but the result of some as yet unknown functional requirement. It is consequently a misnomer to call them displaced amacrine cells and the term *interstitial* amacrine cell is preferable. Very recently, interstitial substance P amacrine neurons have similarly been found in the retina of a New World monkey, the squirrel monkey (Brecha et al. 1982).

Somatostatin fibres have previously been observed exclusively in the inner plexiform layer. However, we have in this study occasionally seen such fibres reaching also the outer plexiform layer of cow, pig, cat, rat and frog retina. In addition, fibres connecting the inner plexiform and outer plexiform layers were at times seen and since we have found somatostatin cell bodies only in the innermost part of the inner nuclear layer it is possible that they represent interplexiform cells. Interplexiform cells are now known in several species (Ehinger 1982) but peptides have previously not been associated with them.

The number of somatostatin fibres seen in the outer plexiform layer was quite small. This contrasts to the dense supply of somatostatin fibres seen by Krisch and Leonhardt (1979) in the outer plexiform layer in the rat retina. The discrepancy in results may be due to differences in specificity or potency of the antibodies used.

Neuropeptides are in some brain neurons known to coexist with conventional neurotransmitters (Hökfelt et al. 1980; Johansson et al. 1981). Since dopamine, an indolamine, GABA, glycine, aspartate, glutamate and acetylcholine occur in the retina which is strictly stratified it is already at this stage feasible to analyse a possible coexistence. Glycine and GABA neurons are not well stratified and are therefore not likely to coexist with any of the neuropeptides studied. Neurons containing acetylcholine, dopamine and indolamine are stratified and known to exist in many species (Ehinger 1982). However, a careful comparison of the number of sublayers in the inner plexiform layer and their positions in the various species does not suggest that somatostatin or VIP coexists with any of these neurotransmitters.

Only very little is known concerning the possible functions of somatostatin and VIP in the retina. VIP has recently been shown to stimulate retinal adenylate cyclase (Watling and Dowling 1981; Longshore and Makman 1981; Schorderet et al. 1981) suggesting an influence on dopaminergic neurons or neurons with dopaminergic adenylate cyclase linked receptors. There are no reports on the effect of somatostatin on the retina. However, the localization of the somatostatin and VIP neurons suggests that they have some modulating influence on the signal traffic, but the available data do not permit any further suggestions.

Apart from somatostatin and VIP several other peptides have been found in the retina with immunohistochemical methods: enkephalins, neurotensin, glucagon, substance P and cholecystokinin (see Ehinger 1982; Karten and Brecha 1982). The neurons containing these neuropeptides are with few

exceptions of the amacrine type, also when the peptides are of potentially excitatory character. Their general morphology and distribution are similar to that of somatostatin- and VIP neurons seen in this study. More than a dozen neuroactive substances have thus to date been found in amacrine cells so that the morphological diversity is matched by a similar biochemical variability. This predicts a corresponding multiplicity and diversity in the circuit functions in the inner plexiform layer.

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VIP neurons have also been demonstrated in chicken retina by Fukuda et al. (1981) (*Current Eye Res* 1:115-118)

GLUCAGON IMMUNOREACTIVE NEURONS IN THE RETINA OF DIFFERENT
SPECIES

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KEY WORDS Neuropeptides, glucagon, retina, immunohistochemistry, amacrine cells, goldfish, frog, pigeon, rat, rabbit, cat, pig, cow.

ABSTRACT Neurons displaying glucagon immunoreactivity were detected among the amacrine cells in the retina of goldfish, frog and pigeon. Nerve cell bodies were located in the inner nuclear layer with their processes ramifying in 2-3 more or less well-defined sublayers in the inner plexiform layer. The distribution of cell bodies and processes varied with the species. In pigeon retina two separate populations of glucagon immunoreactive neurons were found among the amacrine cells. In frog retina glucagon immunoreactivity was also discerned in cell bodies in the ganglion cell layer. These cell bodies sent processes outwards to the inner plexiform layer. No glucagon immunoreactive neurons were detected in the retina of the rat, rabbit, cat, pig or cow.

INTRODUCTION

During recent years immunohistochemical and other immunochemical methods have revealed peptides such as substance P, enkephalins, somatostatin, neurotensin, VIP (vasoactive intestinal polypeptide), glucagon and cholecystokinin in neuronal elements in the central and peripheral nervous systems (Hökfelt et al. 1980; Håkanson et al. 1981) and in the retina (review Ehinger 1982; Karten and Brecha 1982; Tornqvist et al. 1982). Since glucagon immunoreactive neurons have previously been described only in the retina of chicken (Tornqvist et al. 1981) and pigeon (Karten and Brecha 1982) and since considerable species differences have been found for other putative neurotransmitters we have extended the investigation of neurons displaying glucagon immunoreactivity in the retina to the goldfish, frog, pigeon, rat, rabbit, cat, pig and cow.

MATERIALS AND METHODS

Eyes were obtained from goldfish (Carassius auratus), frog (Rana temporaria), pigeon (Columba livia), rat, rabbit, cat, cow and pig, (at least four of each species). Goldfish, frogs and pigeons were decapitated, rats were killed by an overdose of diethyl ether, rabbits by air embolization and cats by exsanguination under pentobarbital anaesthesia. Eyes from cows and pigs were obtained in a nearby slaughter-house within 2-3 min of the slaughter. The retinae were rapidly dissected out and fixed by immersion in ice-cold 0.1 M phosphate buffer containing 4% paraformaldehyde (pH adjusted to 7.2 with 1N NaOH) for 24 h. They were then rinsed repeatedly in 0.1 M phosphate buffer containing 30% sucrose for 24-48 h. The specimens were thereafter frozen on dry ice and sectioned (15 μ m) in a cryostat at -20°C. The sections were processed for the immunohistochemical demonstration of glucagon using

the indirect immunofluorescence technique (Coons et al. 1955). Each section was exposed to glucagon antiserum which was diluted at 1:160 for 3 h at room temperature in a moist chamber. After thorough rinsing in 0.01 M phosphate buffered saline (pH 6.9-7.1) the site of antigen-antibody reaction was revealed with fluorescein isothiocyanate labelled sheep anti-rabbit Ig G (Dako, Copenhagen, Denmark) that was diluted at 1:20, and used for 30 min at room temperature. The antisera contained 0.25% human serum albumin and 0.25% Triton X-100. Immunofluorescence was then observed in a fluorescence microscope with filters selected to give peak excitation at 450-490 nm and barrier filters with a cut-off edge at 515 nm (Leitz I2 filter system). The glucagon antiserum (code 7811) was obtained from Milab, Malmö, Sweden. It has previously been successfully used for demonstrating glucagon immunoreactive neurons in the rat central nervous system (Lorén et al. 1979) and the chicken retina (Törnqvist et al. 1981). The antiserum has been characterized. It is known to cross react with gut type glucagon (glicentin, Lorén et al. 1979), but not with any other known neuropeptide. Nevertheless, it cannot be excluded that the antiserum may also demonstrate other peptides containing the immunoreactive sequence of amino acid residues. However, for the sake of brevity we will refer to the neurons detected in this study as glucagon neurons. A peptide with chromatographical properties identical with those of glucagon has been extracted from chicken retina (Törnqvist unpublished work) which supports the use of this terminology. For controls, the antiserum was exposed to antigen in excess (10-100 µg/ml diluted antiserum) for 24 h and applied to tissue sections as described. These controls had negative results.

RESULTS

Goldfish. A few rounded or slightly polyhedral glucagon cell bodies were found in the innermost cell row of the inner nuclear layer. Their size was approximately that of the surrounding non-fluorescent cell bodies. Their processes formed a sublayer of fine, varicose nerve fibres in sublamina 1 in the inner plexiform layer (Fig. 1). At times short nerve fibres were also seen in the middle of this layer and, rarely, radial nerve fibres connected the two sublayers. The number of glucagon cell bodies in the goldfish was smaller than in the pigeon or frog.

Frog. Rounded or slightly polyhedral cell bodies were found in the innermost cell row of the inner nuclear layer forming a small percentage of the cells in this row. They had approximately the same size as the other cells in their cell row. The cell bodies sent processes to a plexus of nerve fibres in the outermost third of the inner plexiform layer. At times two distinct sublayers with radial communications between them could be discerned within this plexus. Radial nerve fibres also projected inwards through the inner plexiform layer joining a single nerve fibre layer in sublamina 5 (Fig. 2). Now and then glucagon cell bodies were also observed in the ganglion cell layer, sending processes outwards to the nerve fibre sublayers previously described (Fig. 3). They were never observed to send nerve fibres to the optic nerve.

Pigeon. Two types of glucagon cell bodies were seen. The most frequent type was rounded or slightly polyhedral and about the same size as the surrounding non-fluorescent cells. They were situated in the innermost cell row of the inner nuclear layer. These cell bodies sent processes to a continuous nerve fibre layer in sublamina 1 as well as to a discontinuous layer

in sublamina 3 in the inner plexiform layer (Fig. 4). Occasionally, these nerve fibre layers were connected by radially running fibres. The other, more infrequently occurring type of cell body was more elongated and about 3 times larger than the first. It displayed stronger immunofluorescence than the smaller type and its axon hillock was readily visible. These cells sent strongly fluorescent, coarse nerve fibres to three sublayers situated in sublaminae 1, 3 and 5 of the inner plexiform layer (Fig. 5). Altogether the glucagon cell bodies constituted a small percentage of the total number of cell bodies in their cell row (Fig. 6).

Rat, rabbit, cat, pig and cow. In these species no glucagon neurons were detected though sections from them were processed simultaneously and in parallel with sections from the goldfish, frog and pigeon and with the same antibody.

DISCUSSION

Glucagon cells have previously been described in the retina of chicken (Tornqvist et al. 1981) and pigeon (Karten and Brecha 1982). In both species they seem to belong to the group of amacrine cells. Their processes are restricted to two more or less well defined nerve fibre layers in sublaminae 1 and 3 of the inner plexiform layer. However, there is one feature in their morphology that has not been pointed out before. In the pigeon retina two separate populations can be distinguished. One is more common and similar to the cells seen in the chicken retina, while the other is rare with cell bodies about 3 times as big as the first type and with coarse processes ramifying in sublaminae 1, 3 and 5 of the inner plexiform layer. This large cell type has also recently been observed by Brecha and Karten (personal communication, 1982) and in whole mount preparations these cells were seen to send

very long processes to the ora serrata where they formed a circular fibre bundle parallel to the ora.

In the goldfish retina the number of glucagon cell bodies is very low although both the localization and distribution of their nerve fibres resemble the glucagon neurons seen in both the chicken and pigeon.

In the frog retina the number of cell bodies is larger and the pattern of stratification different from that seen in the other species where glucagon processes are restricted to a few narrow sublayers. In the frog they occur throughout the outermost third of the inner plexiform layer. This corresponds roughly to the OFF sublamina (or sublamina a) as defined by Famiglietti and Kolb (1976) but since the physiological effects of glucagon have not been investigated in frog retina the significance of this distribution remains unknown. Moreover, in the frog glucagon cells are found in the ganglion cell layer, an observation unique to this species. These cells have not been observed to send nerve fibres to the optic nerve and it is therefore likely that they represent displaced amacrine cells rather than ganglion cells.

The glucagon cells are thus found in retinae from birds and cold-blooded animals but not in the mammalian retina. They seem to constitute a group of stratified amacrine cells with a pattern of stratification which is similar in goldfish, chicken and pigeon (the smaller cell type). The larger glucagon cells in pigeon and glucagon neurons in frogs have their processes distributed in a somewhat different manner.

Other peptides such as substance P, VIP and somatostatin have been reported in interstitial amacrine cells and interplexiform cells (Brecha et al. 1982; Tornqvist et al. 1982),

but no such cells displaying glucagon immunoreactivity have been observed.

Co-occurrence of two peptides within the same neuron or co-occurrence of peptides and conventional neurotransmitters is known in the central nervous system (Johansson et al. 1981). Immunohistochemistry has shown somatostatin and substance P in neurons in goldfish retina (Yamada et al. 1980; Brecha et al. 1981b), somatostatin and cholecystokinin in frog retina (Osborne et al. 1981; Tornqvist et al. 1982), and enkephalins, substance P, neurotensin, somatostatin and VIP in pigeon retina (Brecha et al. 1979, 1981a; Karten and Brecha 1980, 1982). However, careful comparison of the distribution, number and shape of glucagon neurons with the other peptide containing neurons found in the different species reveals that it is unlikely that any of the peptides co-exist with glucagon within a single neuron.

A similar comparison with other transmitters known to occur in the retina such as dopamine, 5-HT or a related indoleamine, acetylcholine, GABA or glycine reveals that glucagon is not likely to co-occur with any of these either. GABA and glycine neurons are not well stratified whereas dopamine, 5-HT or acetylcholine are stratified but the pattern of stratification is different from that of the glucagon neurons (Ehinger 1982).

The function of glucagon neurons remains unknown. Schorderet et al. (1981) have recently shown that glucagon stimulates retinal adenylate cyclase in the pigeon but not in the rabbit retina. These results suggest an influence on dopaminergic neurons or neurons with adenylate cyclase linked

receptors. Furthermore, the localization of glucagon neurons suggest that they have some modulating influence on the signal traffic, but the data available do not permit further suggestions.

As already noted, several peptides have been demonstrated in retinal neurons: glucagon, VIP, somatostatin, enkephalins, neurotensin, substance P and cholecystokinin. Also when the peptides are of a potentially excitatory character these neurons are with only few exceptions localized among the amacrine cells. More than a dozen putative neurotransmitters have thus to date been demonstrated in amacrine cells suggesting a great variability in their influence on the signal traffic through the inner plexiform layer.

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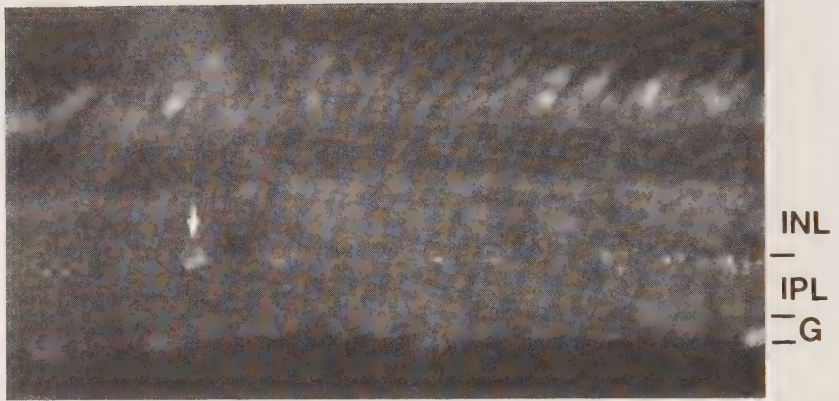


Fig. 1. Glucagon immunoreactive cell body (arrow) in goldfish retina situated in the innermost cell row of the inner nuclear layer. It sends processes to a nerve fibre layer in sublamina 1 of the inner plexiform layer. INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Fluorescence micrograph X 280

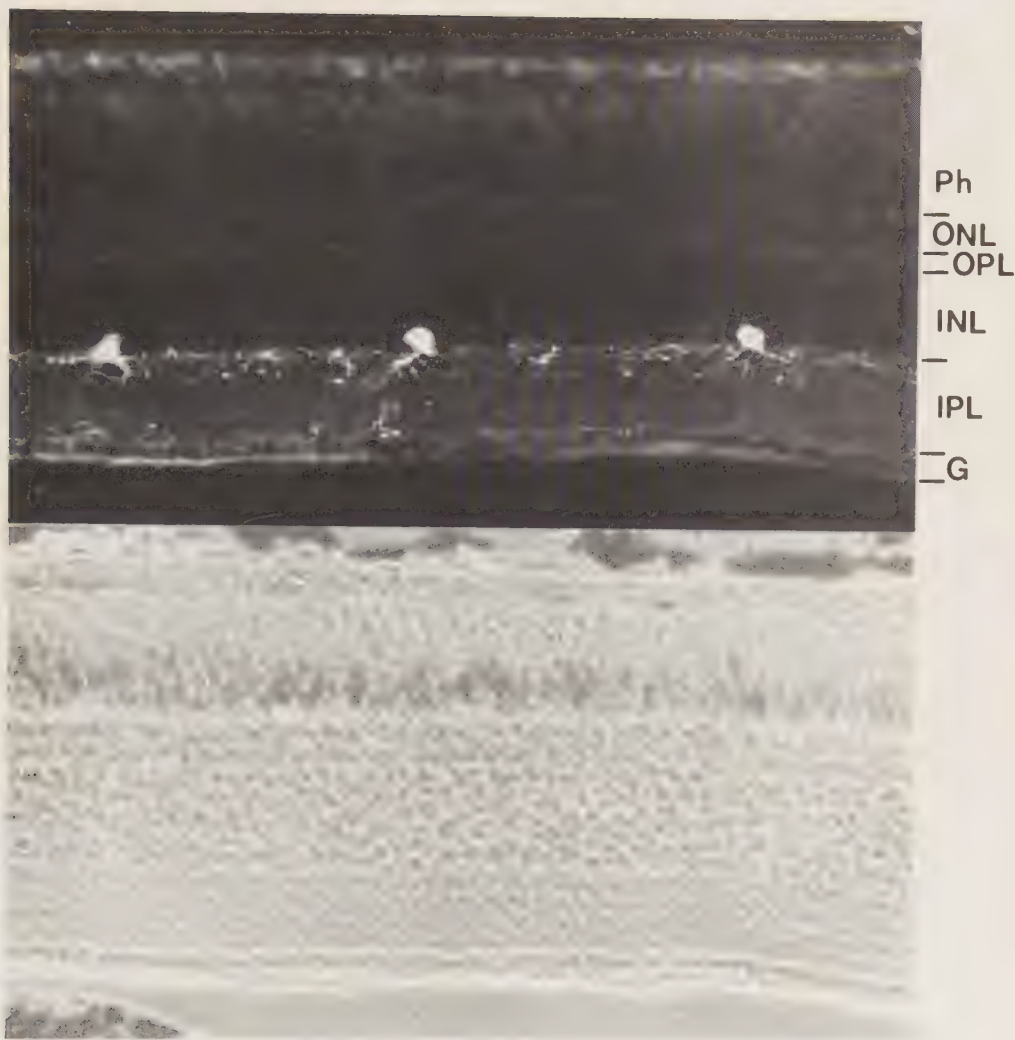


Fig. 2. Glucagon immunoreactive neurons in frog retina. Cell bodies are situated in the innermost cell row of the inner nuclear layer and send processes to the outermost and innermost parts of the inner plexiform layer. Ph, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Above, fluorescence micrograph; below phase contrast micrograph X 280

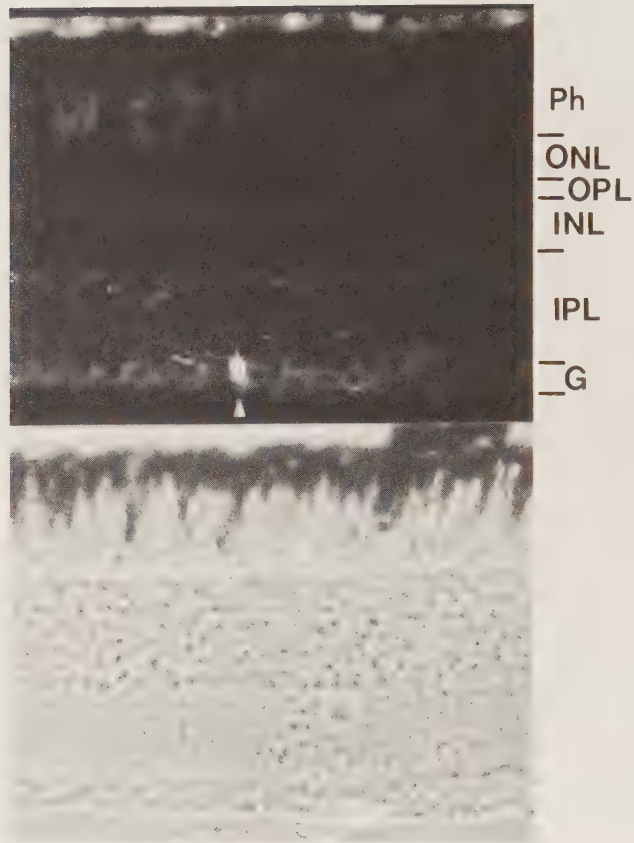


Fig. 3. Glucagon immunoreactive neuron in frog retina. The cell body (arrow-head) is situated in the ganglion cell layer and sends processes outwards to the sublayers in the inner plexiform layer. Designation of layers as in Fig. 2. Above, fluorescence micrograph; below, phase contrast micrograph X 280



Fig. 4. Glucagon immunoreactive neurons in pigeon retina. The cell body shown is of the most frequently occurring type and situated in the innermost cell row of the inner nuclear layer. Processes form a continuous nerve fibre layer in sublamina 1 and a discontinuous one in sublamina 3 (arrow-heads). Designation of layers as in Fig. 1. Fluorescence micrograph X 280

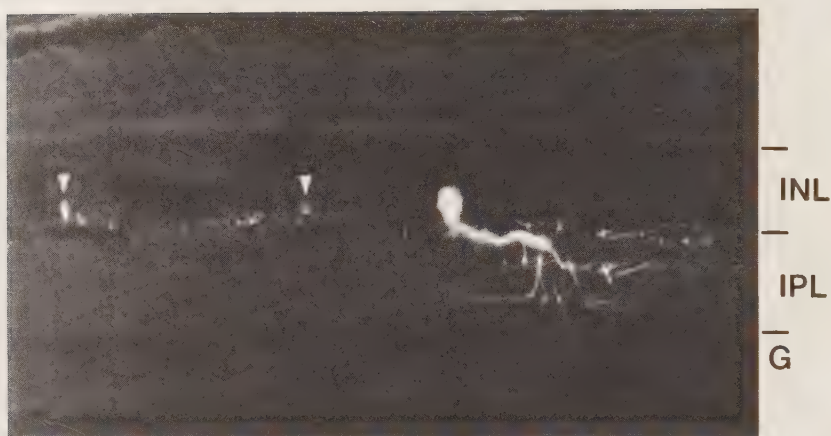


Fig. 5. Glucagon immunoreactive neurons in pigeon retina. Both types of neurons are shown. To the left two cell bodies of the same type as shown in Fig. 4 (arrow-heads), to the right the larger type of cell body sending processes to sublaminae 1, 3 and 5 of the inner plexiform layer. Designation of layers as in Fig. 1. Fluorescence micrograph X 180



Fig. 6. Low power micrograph illustrating the distribution of glucagon immunoreactive neurons in pigeon retina. The continuous nerve fibre layer in sublamina 1 of the inner plexiform layer and cell bodies of the frequently occurring type are seen. Designation of layers as in Fig. 1. Above, fluorescence micrograph; below, phase contrast micrograph X 180

SIMULTANEOUS IMMUNOHISTOCHEMISTRY AND AUTORADIOGRAPHY OF
PEPTIDES AND 5-HYDROXYTRYPTAMINE IN BIRD RETINA

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KEY WORDS Neuropeptides, somatostatin, glucagon, substance P, 5-hydroxytryptamine, immunohistochemistry, autoradiography, retina, pigeon, chicken.

SUMMARY A method was developed for the simultaneous demonstration of two specific cellular constituents. Cryostat sections of formaldehyde fixed tissue from retinæ treated with (^3H)-5-hydroxytryptamine were subjected to immunohistochemistry and subsequent autoradiography. The method takes advantage of the very efficient and specific uptake mechanism that many types of neurons possess which makes it possible to label them with radioactivity.

With this method a study was made on the possible co-occurrence in the avian retina of 5-hydroxytryptamine on one hand and somatostatin, glucagon and substance P on the other. Substance P and 5-hydroxytryptamine were investigated in pigeon retina whereas 5-hydroxytryptamine and somatostatin or glucagon were investigated in chicken retina. Though a large number of cell bodies were examined no co-occurrence of 5-hydroxytryptamine and any of the peptides was found. The sensitivity of the method allows an assertion that if present, double labelled neurons are likely to number less than 0.5-1% of the respective populations.

INTRODUCTION

The retina contains several different neuronal systems which produce and store different putative neurotransmitters such as acetylcholine, dopamine, an indoleamine, gamma-aminobutyric acid (GABA) and glycine (Ehinger 1982). The indoleamine is most probably 5-hydroxytryptamine in birds and cold-blooded animals but not in mammals (Tornqvist et al. 1982 b). These transmitters are all found in amacrine cells and so are, with few exceptions, a number of peptides such as enkephalins, substance P, VIP, glucagon, somatostatin, neurotensin, and cholecystokinin (Eriksen and Larsson 1981, Fukuda et al. 1981, a,b, Tornqvist et al. 1981, Osborne et al. 1981, Kuwayama et al. 1982, Ishimoto et al. 1982, Osborne et al. 1982, Tornqvist et al. 1982, a, c, review: Brecha and Karten 1982).

It cannot be excluded that some of the various neurotransmitters coexist within the same neuron as has been shown in the brain. For example, 5-hydroxytryptamine and substance P occur together in certain neurons in the rat brain stem (Hökfelt et al. 1978). Also TRH and GABA have been proposed to coexist with 5-hydroxytryptamine in the rat central nervous system (Johansson et al. 1981, Nanopoulos et al. 1982). We have therefore developed a method that with a high degree of precision will identify any neuronal perikaryon with two specified neurotransmitters. The procedure takes advantage of the PAP immunohistochemical method and the fact that many neuronal types can be labelled with their transmitter in radioactive form. Basbaum et al. have very recently (1982) published a similar method. However, it is intended for electron microscopy and considerably more complicated and capricious than the one presented here.

MATERIALS AND METHODS

Adult pigeons (*Columba livia*) and chicken (6-8 weeks) received an intraocular injection of 40 μCi and 35 μCi (^3H)-5-hydroxytryptamine respectively. The animals were killed by decapitation 4 hours later. Retinae were rapidly dissected out and fixed by immersion in ice-cold 4% formaldehyde (40 g paraformaldehyde dissolved in 1 l 0.1 M phosphate buffer, pH adjusted to 7.2 with 1 N NaOH) for 24 hours. The specimens were then rinsed for 24-48 hours in 0.1 M phosphate buffer containing 30% sucrose, frozen on dry ice and sectioned in a cryostat at -20°C . The sections (15 μm) were then processed for the immunohistochemical demonstration of the different peptides using the peroxidase-antiperoxidase procedure (Sternberger 1979). Sections were exposed to the peptide anti-serum overnight at $+4^\circ\text{C}$. After rinsing in phosphate buffer they were incubated with unlabelled sheep anti-rabbit IgG for 30 min at room temperature followed by incubation for 30 min with PAP complex, i.e. horseradish peroxidase bound to a rabbit antibody raised against peroxidase. The enzyme was developed by incubation with a solution of 0.05% diaminobenzidine hydrochloride and 0.01% hydrogen peroxidase in Tris buffer (pH 7.6) for 1 h. For autoradiography the unmounted sections were then covered with stripping film (Kodak AR 10) floated on distilled water and exposed for 4, 6, 8 or 12 weeks. The emulsion was developed in Kodak D 19 developer and fixed in Kodafix solution.

Estimates of maximum probable frequencies of double labelled cells were made with statistics based on the binomial distribution. The risk that a cell with co-occurrence of 5-

hydroxytryptamine and a given neuropeptide would have passed undetected was tested by regarding the cells as belonging to either of two groups, those with only one neurotransmitter and those with two transmitters. The risk that, for example, 1% of the population could contain two transmitters and yet have escaped detection could then readily be calculated with a standard program for calculating probabilities according to the binomial distribution.

5-(1,2-(³H)-(N))-hydroxytryptamine, specific activity 500 mCi/mmol, was obtained from New England Nuclear Co., Dreieichenhain, Germany. Glucagon antiserum (code 7811) was obtained from Milab, Malmö, Sweden and used in dilution 1:640. Antisera directed against somatostatin or substance P were purchased from Immuno Nuclear Corporation, Stillwater, Minnesota, USA and used in dilutions 1:500. Unlabelled sheep antirabbit IgG was obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden and used in dilution 1:20 whereas PAP complex was purchased from DAKO immunoglobulins, Copenhagen, Denmark and used in dilution 1:80.

Pilot experiments were performed with all antisera in both chicken and pigeon. The somatostatin and glucagon antisera worked well in either species whereas the substance P antiserum gave technically better results in pigeon. For this reason the more readily available chicken was used for studies on somatostatin and glucagon but pigeon for studies on substance P.

RESULTS

General aspects of the method

Uptake of (^3H)-5-hydroxytryptamine in both chicken and pigeon was found in rounded or slightly ovoid cell bodies in the innermost cell rows of the inner nuclear layer. Radioactivity was also found in two sublayers situated in sublaminae 1 and 4 of the inner plexiform layer. The sublayers were at times connected by labelled processes. Less pronounced labelling was found in cell bodies in the middle of the inner nuclear layer. The background labelling was rather low and no significant chemography was found, neither positive nor negative. The results are in good agreement with previous studies with formaldehyde fluorescence, immunohistochemistry and autoradiography (Florén 1979, Osborne 1982, Tornqvist et al. 1982 b).

In chicken retina somatostatin immunoreactive cell bodies were found in the innermost cell rows of the inner nuclear layer. They were pear-shaped and sent fine varicose processes to a sublayer in sublamina 1 of the inner plexiform layer as well as to a loose plexus of nerve fibres found throughout sublaminae 3 and 4. Glucagon immunoreactive cell bodies were found in the innermost cell row of the inner nuclear layer. They were rounded or slightly polyhedral and sent processes to a continuous nerve fibre layer in sublamina 1 of the inner plexiform layer as well as to a discontinuous one in sublamina 3. In pigeon retina numerous substance P immunoreactive neurons were found in the innermost cell rows of the inner nuclear layer. They sent processes to a sublayer of nerve fibres in sublamina 3 of the inner plexiform layer. The immunohistochem-

ical findings are all in good agreement with previously published results on somatostatin and glucagon in chicken retina (Buckerfield et al. 1981, Tornqvist et al. 1981, Kuwayama et al. 1982) and substance P in pigeon retina (Karten and Brecha 1980). The PAP staining is thus not apparently affected by the development or fixation of the autoradiography. However, the autoradiographic film degrades the optical resolution slightly and may therefore diminish the detectability of very fine nerve fibres such as the somatostatin ones. Cell bodies always remain readily visible.

In the double labelling experiments the PAP label is at the level of the sections whereas the autoradiographic silver grains lie above the sections. With sufficiently high magnification it is easy to reach a decision at the microscope whether a radioactive cell is also PAP stained, provided the grain density is not too high. The differences in colour between the two types of label also helps significantly. However, when taking micrographs, the silver grains not in focus will falsely make a radioactive cell appear dark also when focusing on the PAP stain level. One can avoid misinterpreting the micrographs by noting the edge of the darkened cell body: if the edge is blurred, it is darkened by out of focus grains and hence not PAP positive. If, on the other hand, the edge is sharp, the cell is immunoreactive.

Somatostatin and 5-hydroxytryptamine

In one series of sections 844 somatostatin cell bodies and 321 (^3H)-5-hydroxytryptamine labelled amacrine cell bodies were thoroughly investigated under high magnification ($> \times 600$). The relative proportion of the two cell types can be roughly assessed in Fig. 1. None displayed both positive

PAP staining and uptake of radioactivity (Fig. 2). This corresponds to a 99.7% chance that the true frequency of double labelled cells is less than 0.5% of the frequency of single labelled cells. Cell bodies of the two types were often seen to lie adjacent to one another (Fig. 3). (^3H)-5-hydroxytryptamine labelled cell bodies were in such cases found in the same cell row as the somatostatin cell body or immediately outwards of it. The much more numerous labelled cell bodies in the middle of the inner nuclear layer were simultaneously examined but none showed any PAP staining. In sublamina 1 of the inner plexiform layer somatostatin fibres and the (^3H)-5-hydroxytryptamine sublayer had the same localization whereas in sublamina 4 the (^3H)-5-hydroxytryptamine sublayer corresponded to the innermost part of the loose plexus of somatostatin fibres found throughout sublaminae 3 and 4 (Fig. 4).

Glucagon and 5-hydroxytryptamine

In another series of sections 173 glucagon immunoreactive cell bodies and 380 (^3H)-5-hydroxytryptamine labelled amacrine cell bodies were examined. None of the immunoreactive cell bodies was labelled with radioactivity and none of the radioactive cell bodies displayed any glucagon immunoreactivity (Fig. 5). All cells were examined under high magnification ($> \times 600$). This corresponds to a 99.6% chance that the true frequency of the double labelled cells is less than 1% of the frequency of single labelled cells. Very rarely glucagon and 5-hydroxytryptamine cell bodies were found close to each other. In sublamina 1 both glucagon fibres and (^3H)-5-hydroxytryptamine fibres were found. They were close to each other but it was nevertheless possible to determine that glucagon fibres were always situated outwards of the (^3H)-5-hydroxytryptamine containing fibres (Fig. 5).

Substance P and 5-hydroxytryptamine

No uptake of (^3H)-5-hydroxytryptamine was found in any of 234 thoroughly examined substance P immunoreactive cell bodies. Similarly, 205 (^3H)-5-hydroxytryptamine labelled amacrine cell bodies were examined under high magnification ($> \times 600$) but PAP staining was found in none of them (Fig. 6). This corresponds to a 98.8% chance that the true frequency of double labelled cells is less than 1% of the frequency of single labelled cells. The number of substance P immunoreactive cell bodies and cell bodies in the inner part of the inner nuclear layer that showed uptake of (^3H)-5-hydroxytryptamine was fairly equal. Immunoreactive and radioactive cell bodies were now and then found close to each other but not in any consistent pattern.

DISCUSSION

The procedure developed in this work for the simultaneous demonstration of two putative neurotransmitters in one and the same cell body is based on the fact that neurons using a substance as their transmitter often have the ability to take up this substance also in radioactive form and on the fact that neurons contain enough of their transmitter to enable their demonstration by immunohistochemistry.

The method has some advantages compared to other methods in use for studies of different neuronal populations such as immunohistochemistry with two different stains or serial sectioning. The advantage over the former method is that antibodies are used only to localize one of the cell populations but not the other, thus providing a possibility to examine also neurons which to date cannot be detected with immunohistochem-

ical methods because of lack of antisera. The advantage over serial sections is the possibility to examine both neuronal populations in one and the same section.

The disadvantage with autoradiography is a certain loss of resolution but in the present study this was not disturbing because relevant observations could be made on cell bodies, which are readily resolved. However, a few facts have to be pointed out concerning the procedure. Too heavy radioactive labelling may prohibit detection of positive PAP staining and it is therefore of great importance that different doses of the transmitter labelled with radioactivity are tried and, especially, that different exposure times are used. Likewise, too faint PAP staining may be hidden by overlying silver grains and qualitatively good results of the immunohistochemistry are therefore of greatest importance for the procedure. Provided these precautions are properly attended to and sufficiently high magnification ($> \times 600$) is used the possible co-occurrence of two transmitters in cell bodies is likely to be detected with high precision. The resolution of the autoradiography is not enough for similar assertions in axons or dendrites though the spatial relationship of the terminals can be decided to some extent.

A certain diffusion of radioactivity during fixation and rinsing of the specimen is possible but not likely to affect the results as shown in this study where the results of the autoradiography agreed very well with those obtained with other methods (Florén 1979, Osborne 1982, Tornqvist et al 1982 b). Likewise, chemography did not seem to affect the results. However, thorough rinsing to wash out unreacted formaldehyde was in pilot experiments found of importance both for avoid-

ing chemography and for the quality of the immunohistochemistry.

The method presented thus shows that PAP staining and autoradiography with advantage can be combined on cryostat sections. The procedure should be a good complement to methods already in use for studies of different neuronal populations and possible co-occurrence.

The rich variety of possible transmitter substances within one type of retinal cells, the amacrine cells, has lead to the question whether more than one of these substances are found within the same neuron. Despite the high precision of the present method no such co-occurrence was found. There is little risk that any 5-hydroxytryptamine neurons have escaped detection because the autoradiography with (^3H)-5-hydroxytryptamine in this work gave precisely the frequency and distribution of neurons seen with other methods (Florén 1979, Osborne 1982, Tornqvist et al. 1982 b). The neuropeptides are not always readily demonstrable in cell bodies (e.g. Tornqvist et al. 1982 c) but this is not the case with any of the peptides in the retina of the species investigated. We therefore feel that the procedures used have sufficient sensitivities. Given the high number of cells examined this means that there can be no significant populations of neurons in the retina which simultaneously contain 5-hydroxytryptamine and detectable amounts of either somatostatin, glucagon or substance P. Statistically speaking we can state with more than 98% confidence that the true number of double labelled cells is less than 1% of the studied populations.

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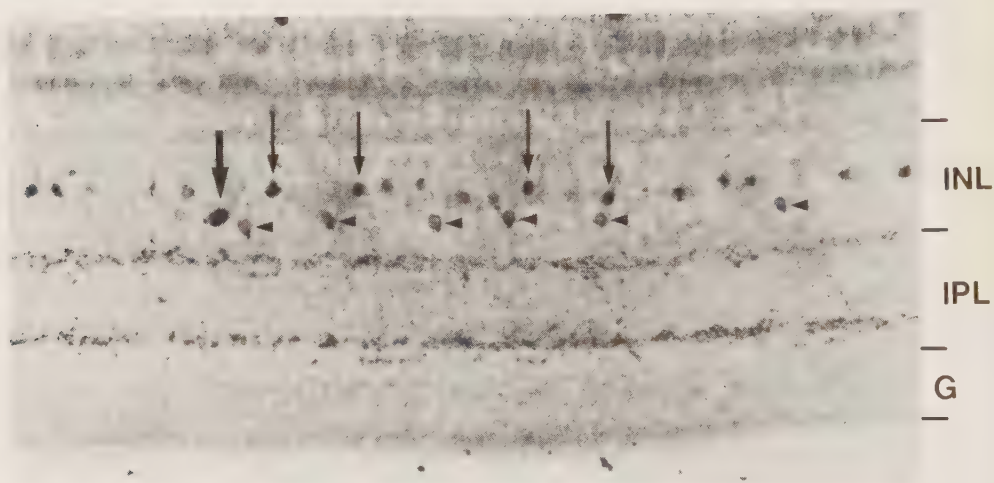


Fig. 1. Low power view of (^3H)-5-hydroxytryptamine labelled and somatostatin immunoreactive neurons in chicken retina. Two types of cell bodies labelled with radioactivity are seen; amacrine cell bodies (large arrow) in the innermost part of the inner plexiform layer and bipolar-like cell bodies (thin arrows) in the middle of the same layer. The somatostatin immunoreactive cell bodies (arrow heads) are more numerous than (^3H)-5-hydroxytryptamine labelled amacrine cell bodies. Somatostatin immunoreactive nerve fibres can not be discerned in the figure whereas two layers of radioactivity are seen in sublaminae 1 and 4 of the inner plexiform layer. INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. X 280.

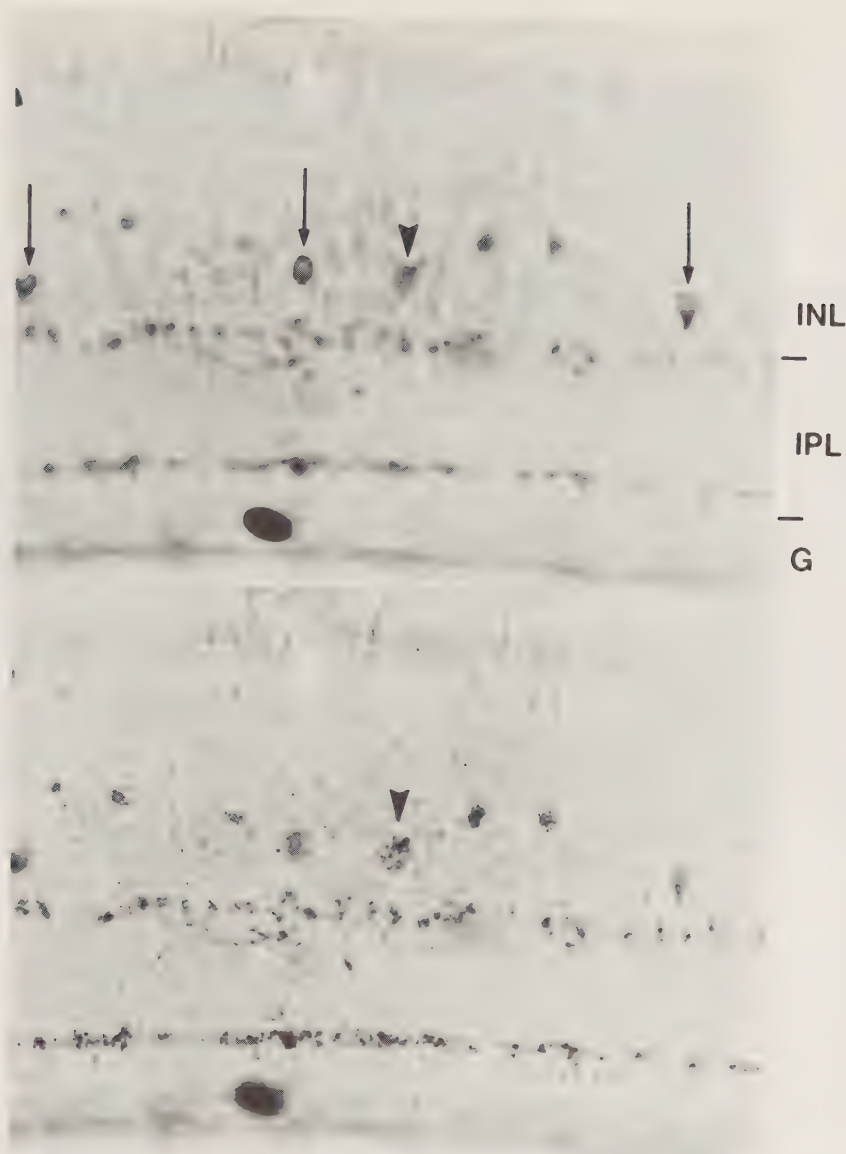


Fig. 2. (^3H)-5-hydroxytryptamine labelled and somatostatin immunoreactive neurons in chicken retina. None of the PAP-positive cell bodies (arrows) are labelled with radioactivity and the cell body labelled with radioactivity (arrow head) does not show any positive PAP-staining. When focus is on the section as in the upper panel this cell body appears dark with a blurred edge indicating that the darkening is caused by the silver grains which are out of focus and not by any immunostaining of the cell body. Designation of layers as in Fig. 1. Above, focus on the section; below, focus on the silver grains. X 450.

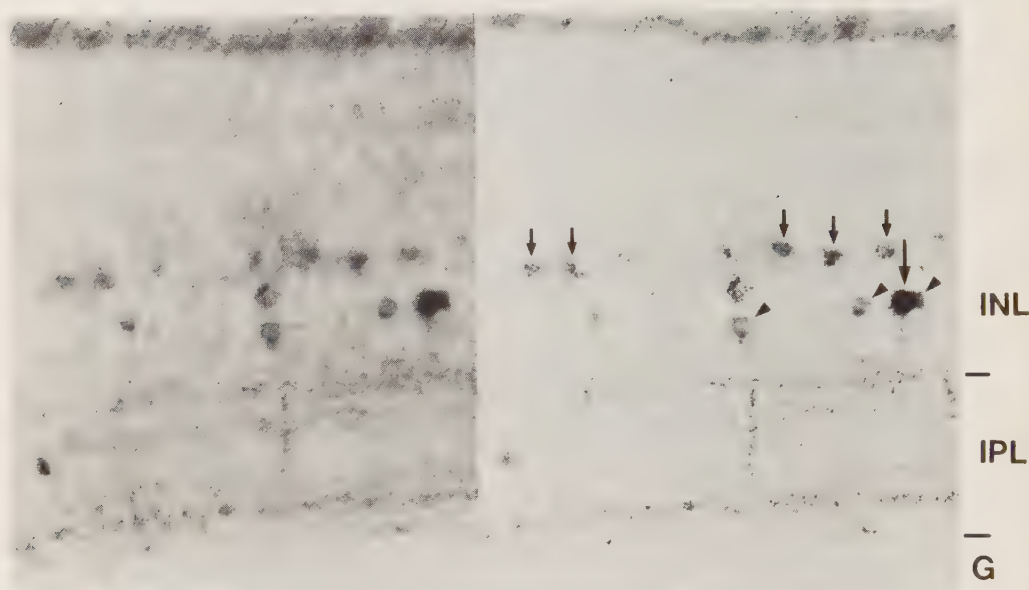


Fig. 3. (^3H)-5-hydroxytryptamine labelled cell bodies in chicken retina situated in the same cell row as or just outwards from the somatostatin immunoreactive cell bodies (arrow heads). The heavily labelled radioactive cell body to the right (large arrow) is situated adjacent to a immunoreactive cell body. The less labelled cell bodies (small arrows) are of the bipolar-like cell type. Designation of layers as in Fig. 1. Left, focus on the section; right, focus on the silver grains. X 475.

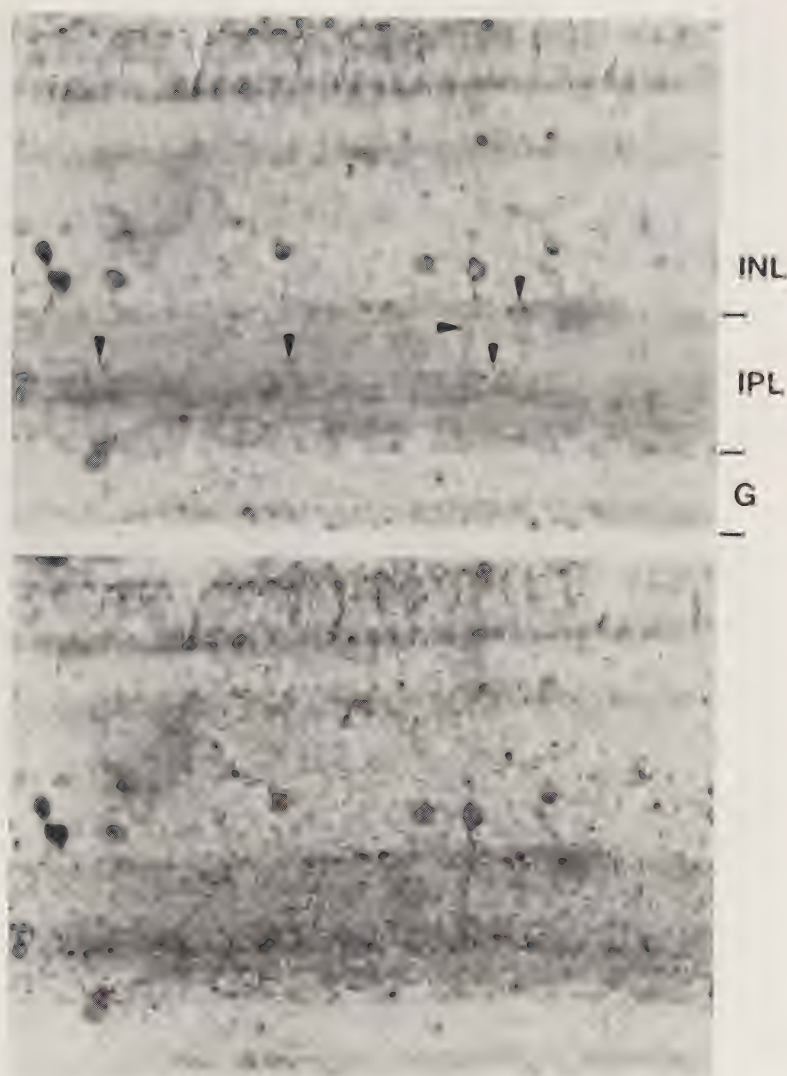


Fig. 4. Somatostatin immunoreactive and (^3H)-5-hydroxytryptamine neurons in chicken retina. In this specimen a lower dose of radioactivity than in Fig. 3 was used which means that the fine somatostatin immunoreactive nerve fibres (arrow heads) in the inner plexiform layer can better be discerned. There is a narrow layer of somatostatin fibres at the border between the inner nuclear and inner plexiform layers as well as a loose plexus of nerve fibres in the innermost part of the inner plexiform layer. Designation of layers as in Fig. 1. Above, focus on the section; below, focus on the silver grains. X 450.

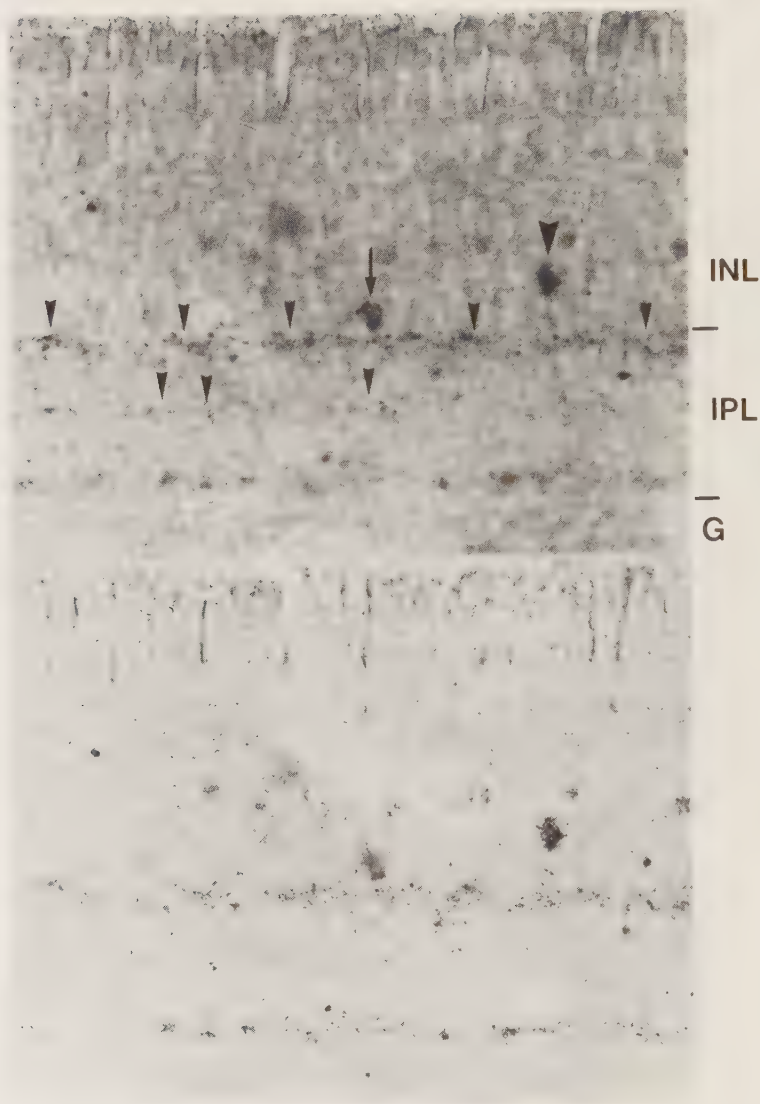


Fig. 5. Glucagon immunoreactive (arrow) and (^3H)-5-hydroxytryptamine labelled (large arrow head) cell bodies in the chicken retina. Glucagon immunoreactive nerve fibres are seen in sublaminae 1 and 3 (small arrow heads) of the inner plexiform layer. The glucagon immunoreactive nerve fibres in sublamina 1 are always situated outwards from the radioactive nerve fibre layer in the same sublamina. Designation of layers as in Fig. 1. Above, focus on the section; below, focus on the silver grains. X 450.

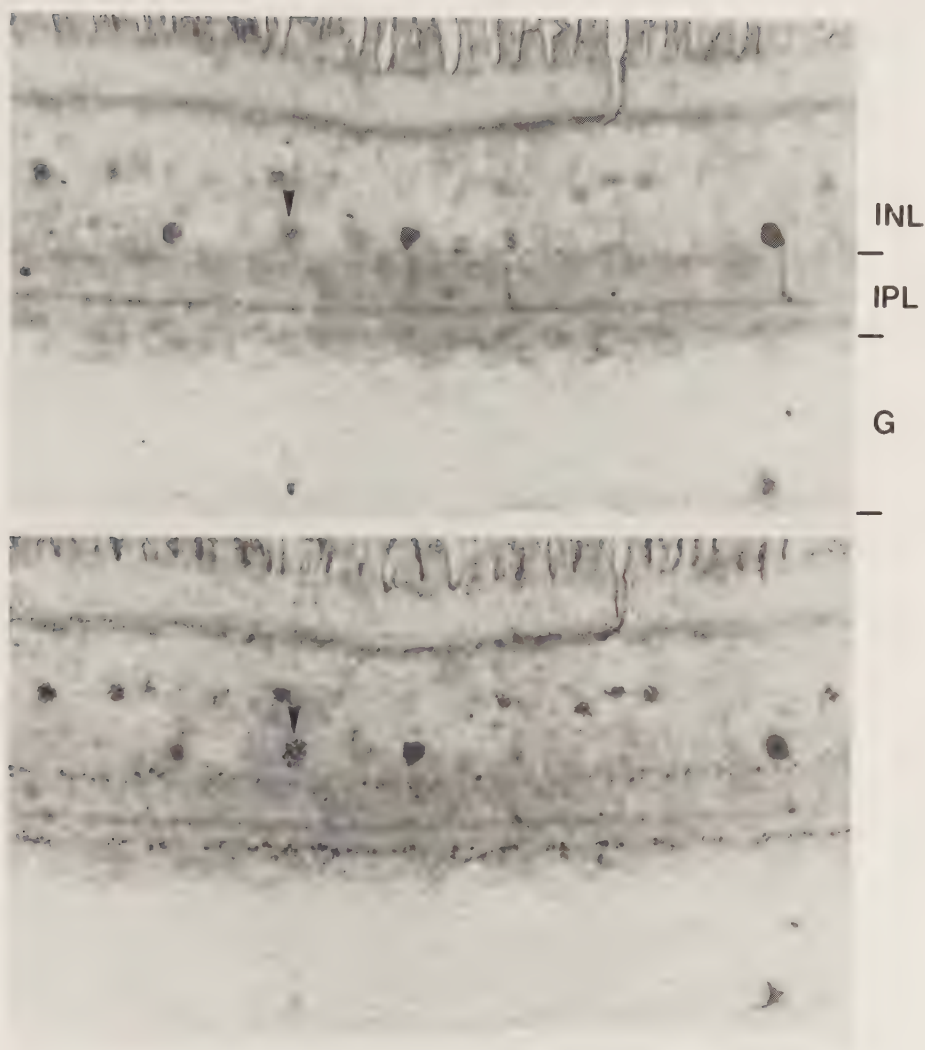


Fig. 6. Substance P immunoreactive and (^3H)-5-hydroxytryptamine labelled neurons in the pigeon retina. None of the four immunoreactive cell bodies in the micrograph above shows any radioactive labelling and no PAP-staining is seen in the radioactive cell body (arrow head). The latter appears dark in the top panel because its associated silver grains which are out of focus. Its blurred edge indicates there is no immunostaining. Designation of layers as in Fig. 1. Above, focus on the section; below, focus on the silver grains. X 450.

GLUCAGON AND VIP IN THE RETINA

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KEY WORDS Neuropeptides, glucagon, vasoactive intestinal polypeptide (VIP), retina, gel chromatography, high performance liquid chromatography (HPLC), radioimmunoassay, cow, pig, cat, rabbit, guinea-pig, rat, pigeon, chicken, frog, goldfish.

SUMMARY Immunoreactive glucagon and immunoreactive VIP have been demonstrated in neuronal elements in the retina of a number of species by immunohistochemistry. In the present study the concentrations of glucagon-like and VIP-like material in retinae from different species were determined by radioimmunoassay. The retinal concentration of glucagon-like immunoreactivity was 10-35 pg/mg in goldfish, chicken, pigeon, rabbit, and frog, whereas retinae from cow, pig, rabbit, and rat contained very little. Retinae from the latter four species were on the other hand rich in VIP-like material whereas retinae from cat, guinea-pig, and goldfish contained very little. The glucagon-like immunoreactive material in chicken and frog retina was characterized by gel chromatography and high performance liquid chromatography (HPLC). The results indicate that the extracted protein is similar to porcine pancreatic glucagon and distinct from porcine glicentin. VIP immunoreactive material extracted from bovine retina could not be distinguished from authentic porcine VIP by gel chromatography.

INTRODUCTION

Several neuropeptides, among them glucagon and vasoactive intestinal polypeptide (VIP), have been demonstrated in retinal neurons in a number of species¹⁻⁸. Glucagon immunoreactive amacrine neurons have been detected in the retina of birds and cold-blooded animals but not in mammals^{1,2,3,8}, whereas VIP has been detected in amacrine cells in several species including mammals⁴⁻⁸. The present study attempts to corroborate previous immunohistochemical findings by quantitating and characterizing the glucagon immunoreactive and the VIP immunoreactive material in extracts from retinæ of several species.

MATERIALS AND METHODS

Retinæ were obtained from cow, pig, cat, rabbit, guinea-pig, rat, pigeon, chicken, frog and goldfish. The animals were all kept in ambient daylight. Retinæ from cow and pig were obtained in a nearby slaughter-house within a few minutes after slaughter. Cats were killed by exsanguination under pentobarbital anaesthesia, rabbits were killed by air embolization, guinea-pigs and rats by an overdose of diethyl ether and pigeons, chicken, frogs and goldfish by decapitation. The retinæ were rapidly dissected out, frozen in liquid nitrogen and weighed before the extraction of glucagon or VIP.

Extraction of glucagon immunoreactive material

Glucagon immunoreactive material was extracted according to Larsson et al.⁹. Retinæ were homogenized in 65% acidified ethanol (1 ml/100mg retina or at least 2 ml) and left at +4°C for 4-12 hours. After centrifugation at 3000 g for 10 minutes the pH of the supernatant was adjusted to 8.0 with NH₄OH. The resulting precipitate was discarded and 1.8 vol ethanol and 3.0 vol peroxide free diethyl ether was added. After 12 hours

at +4°C the precipitate was collected, lyophilized and kept at -20°C until subjected to chromatography or radioimmunoassay in serial dilutions. The acidified ethanol contained Trasylol^(R), 1000 KIE/ml, whereas all other solutions used contained 500 KIE/ml.

Radioimmunoassay of glucagon

Glucagon immunoreactivity in the extracts (0.04 - 0.23g retina) and in the chromatographic fractions was determined by radioimmunoassay as earlier described¹⁰⁻¹². 500 µl of antiserum (code no. 7811, Milab, Malmö, Sweden), diluted 1:60 000, was incubated first with 200 µl standard or tissue extract for 24 hours at +4°C and then for another 72 hours with 500 µl of ¹²⁵I-glucagon at +4°C. Bound and free ¹²⁵I-glucagon were separated by adsorption of free ¹²⁵I-glucagon to dextran-coated charcoal. Each tissue extract was tested in serial dilutions.

Gel chromatography of glucagon-like material

In one series of experiments, extracts (n=3; 0.70-0.85 g retina) from chicken were reconstituted in 3 ml 0.5 M acetic acid and applied to a 1.5 x 35 cm Sephadex^(R) G-50 superfine column equilibrated with 0.5 M acetic acid and eluted with the same solvent at 20 ml/h. The column was calibrated with ¹²⁵I-glucagon. In another series of experiments, extracts from chicken (n=2; 0.34-0.37 g retina) and frog (n=4; 0.40-0.59 g retina) were reconstituted in 3 ml 0.5 M acetic acid. 2 ml samples were then applied to a 1.6 x 100 cm Sephadex^(R) G-50 superfine column, and eluted with 0.5 M acetic acid at 10 ml/h. This column was calibrated with ¹²⁵I-glicentin and ¹²⁵I-glucagon. Fractions of 0.9 ml were collected, lyophilized, and analyzed for glucagon by radioimmunoassay.

High performance liquid chromatography (HPLC)

Extracts from chicken (n=2; 0.83-0.88 g retina) and frog (n=1; 0.56 g retina) were analyzed by HPLC, using an instrument from Waters Ass. (Milford, Mass., USA) with a U6 K injector, two M 6000 A pumps, a 660 solvent programmer, a 440 variable UV-detector and a 3.9 x 300 mm μ -Bondapack C₁₈ column. The solvent was acetonitrile/0.1 M phosphate buffer, pH 3.5, in a 32-50% linear gradient of CH₃CN applied for 20 min. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The concentration of glucagon immunoreactive material in the fractions obtained was determined by radioimmunoassay.

Extraction of VIP immunoreactive material

VIP immunoreactive material was extracted as described by Emson et al.¹³. Retinae were placed in boiling 1 M acetic acid (1 ml/100 mg retina or at least 2 ml) for 10 min, then gently homogenized and centrifuged at 3000 g for 10 min. The supernatant was collected, freeze-dried and stored at -20°C until redissolved and subjected to gel chromatography or analyzed at serial dilutions by radioimmunoassay.

Radioimmunoassay of VIP

Immunoreactive VIP in extracts (0.04 - 0.44 g retina) and chromatographic fractions was estimated using a rabbit antiserum (code no. 7852, Milab, Malmö, Sweden). The antibody is known to recognize the N-terminal 15 amino acid sequence of VIP (Håkanson, unpublished). It does not cross-react with cholecystokinin (CCK), secretin, gastric inhibitory peptide (GIP) or glucagon. VIP was radiolabelled according to the method described by Fahrenkrug and Schaffalitzky de Muckadell¹⁴. 200 μ l of antiserum, diluted 1:16 000, was incubated first with 500 μ l of standard or tissue extract for

three days at +4°C and then with 200 µl (about 4000 cpm) of ^{125}I -VIP for another two days at +4°C. Bound and free ^{125}I -VIP were separated using a second antibody. Each tissue extract was tested in serial dilutions.

Porcine VIP, porcine GIP and porcine secretin were obtained from Peninsula laboratories, San Carlos, USA. Porcine CCK-33 was a generous gift from Prof. V. Mutt, Karolinska Institutet, Stockholm, Sweden. Porcine glucagon was obtained from Novo AS, Bagsvaerd, Denmark and porcine glicentin was a kind gift from Dr A.J. Moody, Novo AS, Bagsvaerd, Denmark. Blue Dextran and Sephadex^(R) G-50 superfine were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Gel chromatography of VIP-like material

Extracts (n=7) of bovine retina (2.3-2.7 g) were subjected to gel chromatography on a 1.6 x 100 cm column of Sephadex^(R) G-50 superfine equilibrated and eluted with 0.25 M ammonium acetate buffer, pH 6.5, containing 72.5 µmol bovine serum albumin/l at +4°C. The flow rate was 10 ml/h and 1.45 ml fractions were collected. The column was calibrated with ^{125}I -labelled VIP. Fractions were analyzed for VIP by radioimmunoassay.

RESULTS

Concentration of glucagon- and VIP-immunoreactive material in retina

The highest concentration of glucagon-like material was found in the frog retina. Pigeon, chicken and goldfish retinae contained less and the least, i.e. very small or even undetectable concentrations, were found in cow, pig, rabbit and rat. The extracts diluted parallel to the standard curve.

TABLE 1

Species	Concentration of glucagon-and VIP-like immunoreactive material in retina			
	number of analyses	Glucagon-concentration [*] pg/mg retina (wet weight) mean $\pm$ S.E.M	number of analyses	VIP concentration ^{**} pg/mg retina (wet weight) mean $\pm$ S.E.M
Cow	4	0.2 $\pm$ 0.01	6	25.4 $\pm$ 3.6
Pig	4	0.4 $\pm$ 0.2	8	15.3 $\pm$ 3.9
Cat			8	0.9 $\pm$ 0.4
Rabbit	6	0.04 $\pm$ 0.04	9	11.6 $\pm$ 3.4
Guinea-Pig			8	0.3 $\pm$ 0.1
Rat	2	not detectable	8	72.4 $\pm$ 15.3
Pigeon	4	19.5 $\pm$ 9.4		
Chicken	10	14.4 $\pm$ 1.2		
Frog	7	35.5 $\pm$ 4.0		
(Rana temporaria)				
Goldfish	6	9.2 $\pm$ 1.2	4	0.8 $\pm$ 0.5
(Carassius auratus)				

* Expressed as equivalents of porcine pancreatic glucagon

** Expressed as equivalents of porcine VIP

The highest concentration of VIP-like material was found in retinae from rat and cow, somewhat lower in retinae from pig and rabbit and the lowest in retinae from cat, guinea-pig and goldfish. The extracts diluted parallel to the standard curve. The results are all presented in Table 1.

Gel chromatography of glucagon and VIP

Fig. 1 shows the gel filtration pattern of the glucagon-like material extracted from frog retina. The major immunoreactive peak had an elution position identical with ^{125}I -glucagon whereas ^{125}I -glicentin had an entirely different elution position. The glucagon immunoreactive material extracted from chicken retina had the same elution position as that extracted from frog retina (not shown in Fig.)

The gel chromatographic elution pattern of the VIP immunoreactive material in extracts from bovine retina is illustrated in Fig. 2. The immunoreactive peak invariably had the same elution position as ^{125}I -labelled porcine VIP.

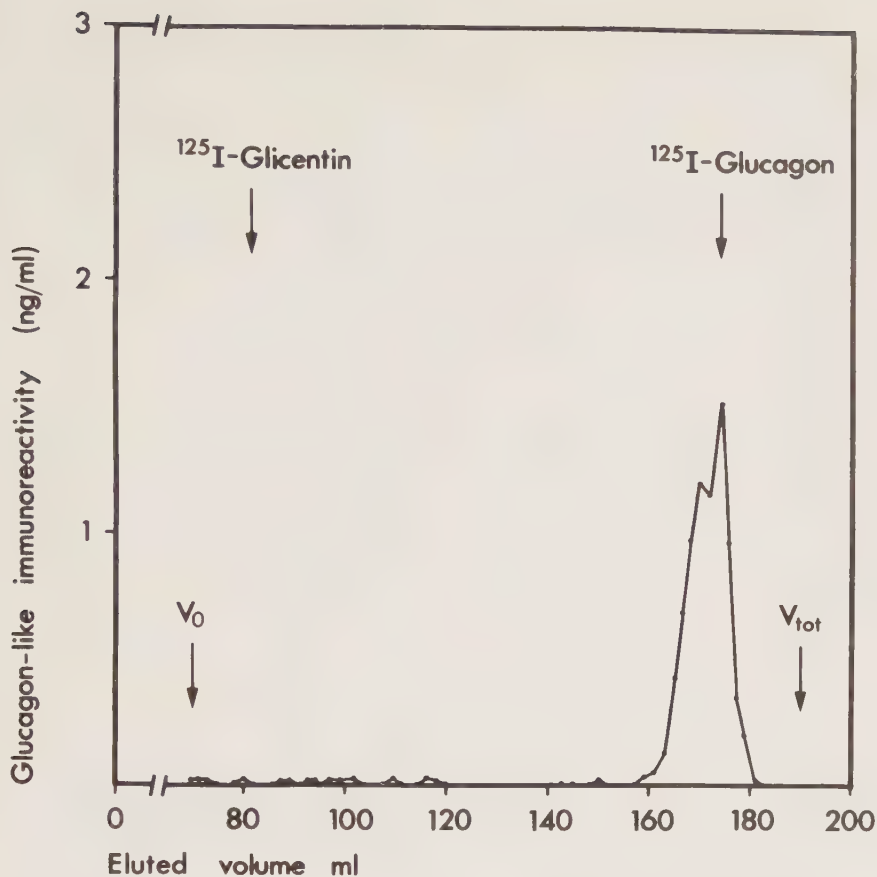


Fig. 1. Gel chromatography of glucagon immunoreactive material extracted from 0.4 g frog retina. Chromatography was performed on a Sephadex^(R) G-50 superfine column. The column was calibrated with Blue Dextran, ^{125}I -labelled porcine glucagon, ^{125}I -labelled glicentin and ^{125}I . Arrows indicate void volume (V_0), total volume (V_{tot}) and elution positions of porcine glicentin and glucagon, respectively. The elution position of glucagon immunoreactive material extracted from chicken retina was identical with that extracted from frog retina (not shown).

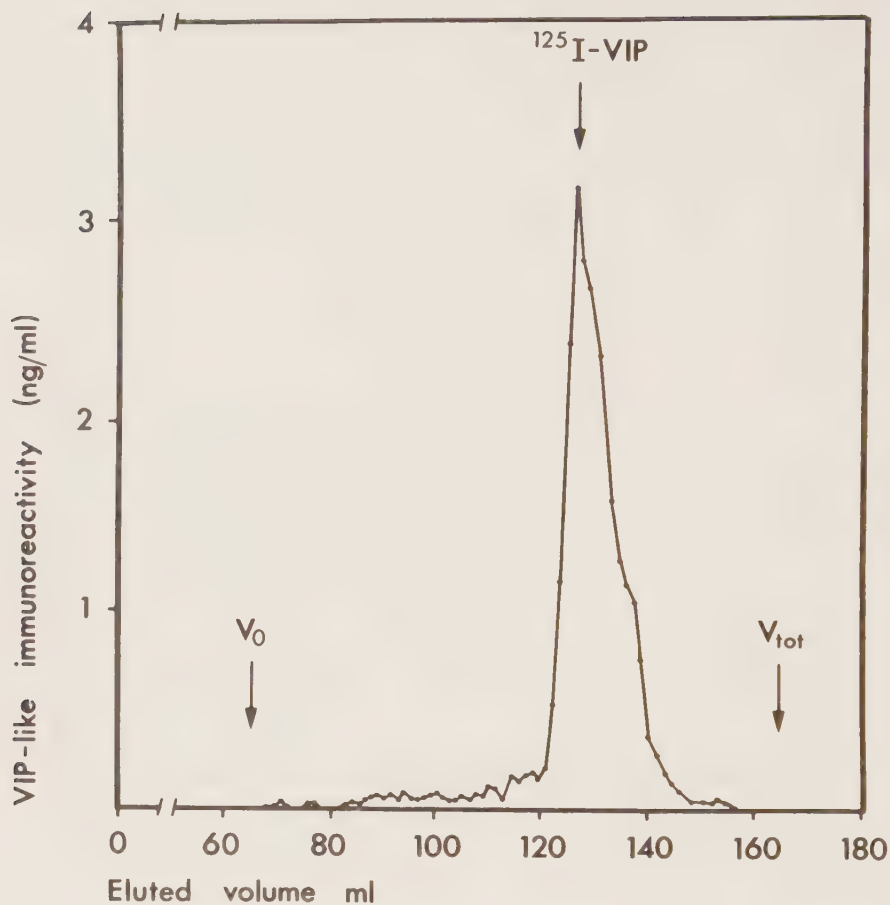


Fig. 2. Gel chromatography of VIP immunoreactive material extracted from 2.3 g bovine retina. Chromatography was performed on a Sephadex^(R) G-50 superfine column. The column was calibrated with Blue Dextran, ¹²⁵I-labelled porcine VIP and ³H-glycine. Arrows indicate void volume (V₀), total volume (V_{tot}) and elution position of porcine VIP.

HPLC analysis

Fig. 3 shows the HPLC elution profile of immunoreactive glucagon in extracts from chicken and frog retina. In both species only one peak with an elution position identical to that of pure porcine glucagon was observed.

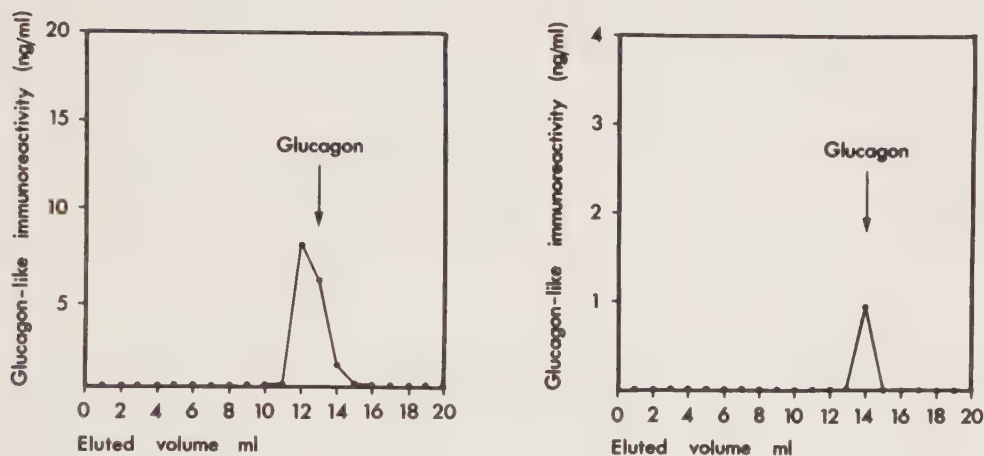


Fig. 3. HPLC profile of glucagon-like immunoreactivity in extracts from 0.84 g chicken retina (left) and 0.56 g frog retina (right). HPLC was performed on a μ -Bondapak C₁₈ column with acetonitrile/0.1 M phosphate buffer as eluant. A linear gradient from 32-50% CH₃CN was applied. There was good agreement between the elution positions of the extracted glucagon-like material and pure porcine glucagon (arrow).

DISCUSSION

Glucagon immunoreactive neurons have been demonstrated in the retina of pigeon, chicken, frog and goldfish^{1-3,8}. These neurons all seem to belong to the group of amacrine cells but their morphology varies from one species to another. Glucagon immunoreactive cell bodies are always found in the innermost cell row in the inner nuclear layer but they are more numerous in frog than in chicken or pigeon and very sparse in the goldfish retina^{1,3}. These cell bodies send processes to nerve fibre sublayers in sublaminae 1 and 3 in chicken and goldfish^{1,3}. In the pigeon retina, an additional nerve fibre sublayer is sometimes seen in sublamina 5^{3,8}. In the frog retina, the pattern of ramification is different with nerve fibres forming a plexus in the outermost third of the inner plexiform layer as well as a single sublayer in sublamina 5³.

The results of the radioimmunoassay with glucagon were in good agreement with previous immunohistochemical findings. The highest concentration of glucagon immunoreactive material was found in the frog retina where the density of glucagon immunoreactive neurons is highest³. In pigeon and chicken the concentration was lower and, correspondingly, glucagon immunoreactive neurons are less numerous in these species^{1,3}. The lowest concentration was found in goldfish retina where cell bodies and nerve fibres are sparse³. Retinae from cow, pig, rabbit or rat contained only very low amounts of glucagon immunoreactive material, which supports previous findings that glucagon immunoreactive neurons are lacking in the mammalian retina^{3,8}.

The identity of the immunoreactive material remains to be established, as the antisera will detect any protein displaying the appropriate antigenic sequence. The glucagon antibody

used in this study and in previous morphological studies^{1,3} is known to cross-react with glicentin (gut-type glucagon) and it was therefore of interest to characterize the immunoreactive material further. Therefore, extracts from frog and chicken retinae were analyzed by gel chromatography and HPLC. With both methods, the elution pattern was in good agreement with that of authentic porcine pancreatic-type glucagon. As judged from the results of the gel chromatography, the immunoreactive material has approximately the same molecular weight as authentic glucagon and is distinct from glicentin which in gel chromatography had a different elution position (Fig.1). Moreover the results of HPLC give good support for the view that the extracted peptide is very similar to glucagon and that the neurons demonstrated contain glucagon rather than glicentin.

As is the case with glucagon, observations on VIP-like material in the retina have so far been mainly restricted to immunohistochemical studies. Thus, VIP immunoreactive neurons have been demonstrated in the retina of baboon, cynomolgus monkey, squirrel monkey, cow, pig, rabbit, guinea-pig, rat, pigeon and chicken^{4-8,15}. In most species, the VIP neurons are localized among the amacrine cells with nerve fibres generally forming two or three sublayers in the inner plexiform layer in a manner that varies with the species investigated⁷. In the baboon retina, VIP neurons are found only in the inner plexiform layer and seem to belong to the group of interstitial amacrine cells⁷.

Quantitative studies on VIP in the retina have previously been performed in the rabbit only¹⁶. However, VIP is demonstrable in many more species. On the whole, the present results are in good agreement with the immunohistochemical find-

ings. Thus the highest concentrations of VIP immunoreactive material were found in rat and cow retinae where the number of VIP neurons is large. In the rabbit retina, VIP neurons are less numerous and, correspondingly, the concentration of VIP-like material was lower and in good agreement with the figures reported by Unger et al.¹⁶. In cat or goldfish retinae, where no VIP immunoreactive neurons have been identified, the amount of VIP-like material was extremely low. Surprisingly enough, only low concentrations of VIP-like material were found in the guinea-pig retina where VIP neurons can readily be demonstrated by immunohistochemistry⁷. The reason for this discrepancy is obscure but the observation may indicate a structural difference between guinea-pig VIP and porcine VIP.

The VIP immunoreactive material in bovine retina co-chromatographed well with standard porcine VIP. Furthermore, the dilution curves of standard VIP and the immunoreactive material extracted from retinae were parallel in the radioimmunoassay procedure, supporting the view that the VIP-like material in the extracts is similar to porcine VIP.

The present study confirms previous immunohistochemical findings that glucagon and VIP are present in the retina. The morphological results strongly suggest that both peptides are located in different subpopulations of amacrine cells¹⁻⁸ and they can therefore be presumed to modulate the transfer of visual information from the photoreceptors to the brain. However, the details of this process remain unknown.

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